OSCILLATORY COMPRESSIVE LOADING EFFECTS ON MESENCHYMAL PROGENITOR CELLS UNDERGOING CHONDROGENIC DIFFERENTIATION IN HYDROGEL SUSPENSION

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3-D	three-dimensional
BAC	bovine articular chondrocyte
$BaCl_2$	barium chloride
BMP	bone morphogenetic protein
$CaCl_2$	calcium chloride
CPM	counts per minute
Dex	dexamethasone
DMEM	dulbecco's modified eagle's media
DMMB	1,9-dimethyl-methylene blue
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
HCl	hydrogen chloride
IGF	insulin-like growth factor
MPC	mesenchymal progenitor cell
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
PLLA	poly(L-lactic acid)
PLGA	poly(DL-lactic-co-glycolic acid)
PBS	phosphate-buffered saline
SEM	standard error of the mean
sGAG	sulfated glycosaminoglycan
TGF-β	transforming growth factor-beta

SUMMARY

Articular cartilage functions to maintain joint mobility. The loss of healthy, functional articular cartilage due to osteoarthritis or injury can severely compromise quality of life. To address this issue, cartilage tissue engineering approaches are currently in development. Bone marrow-derived mesenchymal progenitor cells (MPCs) hold much promise as an alternative cell source for cartilage tissue engineering and other cell-based cartilage repair approaches. While previous studies have established that MPCs from humans and a number of other species undergo *in vitro* chondrogenic differentiation, additional research is needed to define conditions that will enhance chondrogenic differentiation of MPCs, increase matrix production by differentiating cultures, and further support development of functional tissue-engineered cartilage constructs. Mechanical loading may be an important factor regulating chondrogenic differentiation of MPCs and cartilage matrix formation by chondrogenic MPCs. This thesis work evaluated the influence of oscillatory unconfined compressive mechanical loading on *in vitro* MPC chondrogenic activity and biosynthesis within hydrogel suspension.

Loading was conducted using MPCs cultured in media supplements supporting chondrogenic differentiation, thereby enabling the evaluation of distinct MPC cultures based upon the time of loading initiation. Possible interactions between the number of days in chondrogenic media preceding loading initiation and the ability of the MPC culture to respond to mechanical stimulation were explored in two different loading studies. The first loading study investigated the effects of 3 hour periods of daily

oscillatory mechanical stimulation on subsequent chondrogenic activity, where chondrogenic activity represented an assessment of cartilage matrix production by differentiating MPCs focusing on proteoglycan synthesis and sGAG accumulation. This study found that oscillatory compression of MPCs initiated during the first seven days of culture did not enhance chondrogenic activity above the level supported by media supplements alone. The second loading study evaluated changes in biosynthesis during a single 20 hour period of oscillatory mechanical stimulation to assess mechanoresponsiveness of the MPC cultures. This study found that MPCs modulated proteoglycan and protein synthesis in a culture time-dependent and frequency-dependent manner upon application of oscillatory compression. Together the two loading studies provide an assessment of dynamic compressive mechanical loading influences on MPC cultures undergoing chondrogenic differentiation. The information gained through *in vitro* studies of differentiating MPC cultures will increase basic knowledge about progenitor cells and may also prove valuable in guiding the future development of cartilage tissue engineering approaches.

CHAPTER 1

INTRODUCTION

Motivation

Articular cartilage functions to maintain joint mobility by providing a low friction interface under complex cyclic mechanical loading. The loss of healthy, functional articular cartilage due to osteoarthritis or injury results in joint pain and loss of mobility, severely compromising quality of life. Due to its avascularity and relative acellularity, cartilage has a limited capacity for self-repair, both in response to acute injuries and chronic degeneration [1, 2]. Although limited progress has been made, current clinical solutions to repair cartilage damage fail to restore long-term function and alternative cartilage repair strategies are needed.

Tissue engineering approaches for articular cartilage repair are currently in development, with the goal to restore tissue functionality in the damaged regions [3, 4]. These approaches generally involve culturing chondrocytes on three-dimensional (3-D) scaffold materials *in vitro* to produce a cartilage-like tissue that can then be implanted into the defect site [5-8]. While the use of tissue-engineered cartilage holds much promise as a future clinical strategy, the development of this technology is hindered in part by the lack of a sufficient donor cell population. Articular chondrocytes can be isolated from mature tissue, but problems associated with this approach include limited tissue harvest sites, tissue donor site morbidity, and limited expansion potential of the donor population [6, 9].

More recently, mesenchymal progenitor cell populations (MPCs) from tissues including periosteum, bone marrow, and adipose tissue have been shown to contain cells capable of chondrogenic differentiation when exposed to appropriate growth factor stimulation [10-14]. The use of progenitor cells in cartilage tissue engineering may avoid the problems associated with use of mature chondrocytes. Progenitor cells can be more easily harvested, are isolated from tissue that can be replenished or replaced, and have a higher proliferative potential [1]. However, optimal conditions that will enhance chondrogenic differentiation of MPCs, increase matrix production by differentiating cultures, and support development of functional tissue-engineered cartilage constructs have not been defined.

Mechanical loading has been proposed as a stimulatory factor for the *in vitro* development of tissue-engineered cartilage constructs [15-17]. Mechanical loading may be an important factor regulating chondrogenic differentiation of postnatal progenitor cells and cartilage matrix formation by maturing chondroprogenitor cells. The biomechanical environment has been proposed to play a fundamental role in the development and maturation of cartilage [18]. Developmental biology studies support the idea that the biomechanical environment helps guide cartilage formation from the undifferentiated mesenchyme during limb and joint development [19, 20]. Thus, consideration of mechanical loading effects on MPC chondrogenic differentiation, on matrix production by differentiating cultures, and on long-term tissue development may be critical to the *in vitro* development of tissue-engineered cartilage constructs utilizing MPCs. However, studies concerning the specific effects of mechanical stimulation on MPCs seeded within 3-D matrices have been limited.

Thesis Objective

The objective of this thesis work was to evaluate the influence of dynamic mechanical stimulation on *in vitro* chondrogenic activity and biosynthesis of differentiating MPCs within hydrogel suspension. Oscillatory unconfined compressive mechanical loading was chosen as the loading mode of interest based in part upon the primary role of compression in normal repetitive joint loading [21, 22]. Compressive mechanical loading has also been evaluated in previous *in vitro* loading studies with cartilage explants, isolated chondrocytes, and chondroprogenitor populations [19, 23, 24]. To focus on mechanical stimulation of differentiating MPC cultures, loading was conducted using MPCs exposed to media supplements which supported chondrogenic differentiation and loading was initiated during the first week of culture. Altering the culture time for loading initiation enabled comparisons of loading effects on distinct MPC cultures. In evaluating loading effects on MPC chondrogenic activity, this activity represented an assessment of cartilage matrix production by differentiating MPCs focused on proteoglycan synthesis and sGAG accumulation.

Specific Aims

Three specific aims were set forth to address the thesis objective. Guiding the development of these aims was the underlying primary focus of the thesis work, which was to explore possible interactions between the length of culture time (i.e. number of days) in chondrogenic media preceding loading initiation and the ability of the MPC culture to respond to mechanical stimulation. The length of culture time preceding loading initiation would be representative of both cellular changes related to the current differentiation "state" of the cell and extracellular changes resulting from matrix

production by the differentiating MPCs. After defining appropriate conditions for MPC chondrogenic differentiation in Aim 1, Aim 2 investigated the effects of 3 hour periods of daily oscillatory mechanical stimulation on MPC chondrogenic activity. Finally, Aim 3 evaluated changes in biosynthesis during a single 20 hour period of oscillatory mechanical stimulation to assess mechanoresponsiveness of the MPC cultures.

Specific Aim 1: To define culture media conditions that support goat MPC chondrogenic differentiation and subsequent matrix accumulation in hydrogel suspension and to establish a time course for matrix synthesis and accumulation

Although basic chondrogenic media conditions supporting MPC differentiation and matrix accumulation had been previously established, species variability had been noted and a comprehensive evaluation of the effects of specific media components was lacking in the literature. Furthermore, cartilage matrix production by goat MPCs undergoing chondrogenic differentiation had been evaluated only at one or two selected times in previous studies. Therefore, experiments were conducted to evaluate media conditions and to assess the timing of matrix synthesis and accumulation in preparation for the loading studies.

The evaluation of chondrogenic differentiation utilized biochemical analyses and histological analyses to validate the production of the two major structural components of articular cartilage: proteoglycans and type II collagen. Proteoglycan production was assessed quantitatively by determining proteoglycan synthesis, measured as the ^{35}S-sulfate incorporation rate, and sulfated glycosaminoglycan (sGAG) accumulation. Type II collagen production was assessed qualitatively with immunohistochemical staining.

Protein synthesis, measured as the ^{3}H-proline incorporation rate, and DNA content were also evaluated as general culture markers.

The initial culture conditions utilized TGF-β1 and/or dexamethasone, supplementation factors used previously in human MPC differentiation studies, to evaluate effects on chondrogenic differentiation and matrix accumulation [11, 12, 25]. The duration over which supplementation factors were provided (early versus entire culture period) was also evaluated. Criteria for comparison of culture media conditions included the ability to support production of cartilage matrix components and the amount of matrix accumulation.

Specific Aim 2: To evaluate the effects of oscillatory compressive loading on MPC chondrogenic activity

The effects of oscillatory mechanical stimulation on differentiating MPC cultures were evaluated by assessing the chondrogenic activity following application of daily 3 hour loading periods for 3 consecutive days. Since the duration of exposure to chondrogenic media preceding loading initiation could modulate the MPC response to mechanical loading, oscillatory unconfined compressive loading was initiated following a variable preculture period of 1 to 21 days in media containing TGF-β1, a supplement demonstrated to support MPC chondrogenic differentiation. It was hypothesized that dynamic compressive loading in combination with chondrogenic supplements would enhance the chondrogenic activity of MPCs relative to the influence of supplements alone in a preculture time-dependent manner. Proteoglycan synthesis, measured as the ^{35}S-sulfate incorporation rate, and proteoglycan accumulation, measured as sulfated

glycosaminoglycan (sGAG) accumulation, were utilized to assess MPC chondrogenic activity and were supported by the detection of type II collagen expression.

Specific Aim 3: To evaluate MPC biosynthesis during continuous oscillatory compressive loading

The mechanoresponsiveness of MPC/alginate cultures undergoing chondrogenic differentiation was evaluated for a loading protocol shown previously to enhance biosynthesis of chondrocytes in hydrogel suspension [24]. Our *hypothesis* was that oscillatory compressive mechanical loading would stimulate proteoglycan and protein synthesis in differentiating MPC cultures, but would not modulate biosynthesis in prechondrogenic MPC cultures. Oscillatory compressive loading was applied continuously for a single 20 hour period to gels that were being cultured in media containing radiolabeled sulfate (for proteoglycan synthesis) and proline (for protein synthesis) compounds. For all groups, the effects of loading were determined by comparing radiolabel incorporation rates in dynamically loaded constructs to static control constructs. Loading was initiated at culture times (days 1, 4, and 7) demonstrated in Aims 1 and 2 to correspond to distinct MPC cultures, as distinguished by increasing levels of proteoglycan synthesis and sGAG accumulation with increasing time in culture.

3-D MPC Culture System

Culture of goat MPCs in alginate suspension was the standard 3-D culture system utilized in all thesis aims. In selecting the MPC species for the thesis work, long-term directions related to this work were taken into consideration. It was therefore decided to choose an adult nonhuman species that would be appropriate for use as a future *in vivo* cartilage repair model. Selection of the goat was based in part upon the recognition that

this species had been used increasingly for cartilage repair studies in recent years [26-28]. Moreover, *in vitro* chondrogenic differentiation of goat MPCs had been previously demonstrated [29, 30].

Alginate was selected as the hydrogel to be utilized in the thesis experiments. Alginate is a linear polymer composed of β-D-mannuronic acid blocks, α-L-guluronic acid blocks, and mixed blocks linked by glycosidic bonds. A viscous solution of alginate can be converted into a thermally irreversible gel by exposure to multivalent cations. The cations form crosslinks in alginate by binding the guluronic residues, inducing a sol-gel transition in the material, and this crosslinking can be reversed by ion exchange. The selection of alginate was related in part to the long-term application of this work. The 3-D culture system needed to be appropriate for possible future *in vivo* evaluations. Alginate has served as a matrix to deliver chondrocytes and MPCs to cartilage defects [31, 32]. *De novo* cartilage formation has been observed in chondrocyte/alginate constructs implanted subcutaneously in mice [33, 34]. Furthermore, alginate has frequently been used for *in vitro* culture of chondrocytes [35-37]. More recently, alginate hydrogels have been demonstrated to support chondrogenic differentiation of MPC cultures [38-40]. Importantly, alginate has also been used as a culture system for *in vitro* mechanical loading of isolated chondrocytes [41, 42].

Significance

Bone marrow-derived MPCs hold much promise as an alternative cell source for cartilage tissue engineering and other cell-based cartilage repair approaches. The combination of signals present in the cartilage repair environment will influence all aspects of new tissue development resulting from implanted MPCs, from the early stage

of chondrogenic differentiation to the intermediate stage of matrix production by differentiating and maturing cultures to the late stage of long-term tissue growth and integration. The contributions of specific factors to each of these stages cannot be determined within the complex *in vivo* environment. *In vitro* studies are therefore necessary to rigorously evaluate the influence of isolated factors, such as mechanical stimuli, on MPC cultures. The primary concern of this thesis was to evaluate the influence of dynamic mechanical stimulation on MPC chondrogenic activity and biosynthesis, thereby addressing an issue that had received only limited attention in prior experimentation with MPCs. Furthermore, studies were conducted with goat MPCs, providing previously unknown information about a nonhuman MPC population that correlates to a current preclinical animal model of cartilage repair. The studies in aims 1 and 2 contributed to knowledge about factors influencing chondrogenic differentiation and subsequent matrix production by differentiating MPC cultures, while the studies in Aim 3 aided in further characterizing the phenotype of differentiating MPC cultures. The information gained through *in vitro* studies of differentiating MPC cultures will be important for increasing basic understanding of MPCs and may also prove valuable in guiding the future development of cartilage repair approaches.

CHAPTER 2

LITERATURE REVIEW

Hyaline Cartilage: Development and Growth

Hyaline cartilage is a tissue critical to the body's structural framework and function. It serves important roles in joint mobility, as the lining for articulating bone surfaces, and in bone growth and repair, as the scaffold that supports bone formation in endochondral ossification. Chondrocytes are the cellular mediators of cartilage genesis, growth, and maturation. Chondrogenesis involves the commitment of undifferentiated mesenchymal cells to the chondrocyte lineage. This process is critical to appendicular skeletal development and supports cartilage formation leading to endochondral ossification in fracture repair sites. After chondrogenesis and subsequent cartilage formation, chondrocytes follow a distinct differentiation pathway determined by the cartilage type. In growth plate and fracture callus cartilage, chondrocytes undergo proliferation followed by maturation to the terminally differentiated hypertrophic phenotype. Expression of the hypertrophic phenotype is critical to the progression of endochondral ossification. In mature articular cartilage, chondrocytes typically maintain a resting, non-proliferative phenotype. However, in pathological conditions such as osteoarthritis, articular chondrocytes express some characteristics of the hypertrophic phenotype.

During embryogenesis, mesenchymal cells from the limb buds undergo a coordinated process of condensation and chondrogenesis leading to the formation of cartilaginous models of the appendicular skeletal elements [43]. The majority of embryonic limb cartilage is eventually degraded and replaced by bone and marrow elements in a series of

regulated cycles of endochondral ossification [44]. The first wave of endochondral ossification is initiated at the central primary ossification site in the limb and eventually extends toward the limb ends, leading to the appearance of the diaphyseal cavity and the establishment of the epiphyseal growth plates. In early postnatal life, the appearance of the secondary ossification centers in the epiphyseal regions leads to the replacement of epiphyseal cartilage with trabecular bone, while the growth plates remain intact. Endochondral ossification occurs at the terminal hypertrophic zones of the growth plates, leading to longitudinal bone growth. The growth plates persist throughout puberty, but are eventually replaced by bone in the adult. The developmental origins of articular cartilage are less clear and could include contributions from epiphyseal limb chondrocytes and from mesenchymal interzone cells present during joint development [45]. Articular cartilage undergoes growth and zonal organization, leading to the development of a mature tissue present throughout postnatal life [46]. A region of calcified cartilage serves as the interface between articular cartilage and the underlying subchondral bone. The calcified cartilage supports endochondral ossification, leading to bone deposition and remodeling at the interface [47].

Regulation of chondrogenesis during embryonic skeletal development has been studied in both *in vivo* and *in vitro* models. The onset of chondrogenesis is preceded by the condensation of mesenchymal cells, mediated through the interactions of cell adhesion molecules including N-CAM and N-cadherin [43]. Condensation leads to the activation of multiple transcription factors and is thought to promote a cell shape conducive to chondrocyte differentiation directly related to cytoskeletal rearrangement [48, 49]. Present in both chondroprogenitor cells and chondrocytes, Sox-9 has been

proposed to be a master transcription factor for chondrocyte differentiation and may regulate multiple steps in chondrogenesis [50]. It directly supports the expression of genes for the cartilage matrix proteins aggrecan and types II, IX, and XI collagen, while its absence has been shown to block mesenchymal condensation [51, 52]. Multiple factors have been demonstrated to promote *in vitro* chondrocyte differentiation in limb bud cultures and mesenchymal cell lines, including members of the TGF-β and BMP families [53-57].

Articular Cartilage: Importance of Structure and the Biomechanical Environment

Articular cartilage is the hyaline cartilage lining the contacting bone surfaces in joints. It is a dense connective tissue consisting of tissue fluid, an extracellular matrix composed of collagens, proteoglycans, and noncollagenous proteins, and chondrocytes. It acts to resist compression, distribute joint forces, and provide a low-friction bearing surface. The composition and structure of the cartilage tissue directly contribute to tissue function. Tissue fluid comprises up to 80% of the cartilage wet weight and consists of water with dissolved gases, metabolites, and cations. Water in the tissue is pressurized during loading and supports a significant portion of the load. Proteoglycans are composed of a protein core that is associated with glycosaminoglycan chains, which are formed from disaccharides that contain negatively charged sulfate and carboxylate groups. While proteoglycans are present in cartilage in several forms, it is the large, aggregating proteoglycans that notably contribute to tissue hydration and thereby indirectly support the tissue's resistance to compression. Type II collagen is the predominant collagen in articular cartilage and, combined with minor portions of types IX and XI collagen, forms a fibrillar mesh that provides the tissue with tensile strength.

The biomechanical environment plays an important role in the maturation and maintenance of articular cartilage. Articular cartilage has been demonstrated to respond to changes in mechanical loading across the joint. Experimental studies have indicated that exercise leads to increased proteoglycan content in articular cartilage, while joint immobilization leads to a loss of proteoglycan content that can be recovered with restoration of joint mobility [58]. These changes in tissue composition are the result of changes in chondrocyte metabolism. *In vitro* studies have provided fundamental information regarding how mechanical loading influences articular chondrocytes. Static compression of cartilage explants decreased ^{3}H-proline and ^{35}S-sulfate incorporation, while dynamic compression increased ^{3}H-proline and ^{35}S-sulfate incorporation within given frequency and deformation ranges [23, 59, 60].

While cartilage explant culture retains many aspects of the biomechanical environment chondrocytes experience *in vivo*, the complexity of the system makes it challenging to discern how individual factors may influence chondrocyte behavior. To further explore linkages between mechanical stimulation and changes in cellular activities, *in vitro* studies have been conducted on isolated chondrocytes. In studies with chondrocytes suspended in hydrogels, the length of culture time preceding exposure to compressive mechanical loading appeared to influence the cellular response. For gel suspensions subjected to loading after multiple weeks of culture, static compression was found to decrease ^{3}H-proline and ^{35}S-sulfate incorporation, while dynamic loading increased both incorporation rates [24, 41]. For gel suspensions subjected to loading within the first 1-5 days of culture, dynamic loading stimulated ^{35}S-sulfate incorporation, but the modulation of ^{3}H-proline incorporation by dynamic loading was found to be

variable [24, 61, 62]. Conversely, nitric oxide production was inhibited by oscillatory compression in early gel cultures [63]. Hydrostatic pressure and pressure-induced strain stimulated proteoglycan synthesis in chondrocyte monolayers [64, 65]. Type II collagen and aggrecan mRNA levels were stimulated by intermittent hydrostatic pressure, but not influenced by constant pressure, in monolayer cultures [66]. Type II collagen and aggrecan mRNA were inhibited, while nitric oxide production was stimulated by shear stress applied to monolayers [67]. The response of isolated chondrocytes to mechanical loading, in the absence of an extensive extracellular matrix, may have important implications for cartilage repair strategies.

Clinical Repair Strategies for Articular Cartilage

Maintaining the integrity of articular cartilage is crucial to normal joint mechanics, yet cartilage that is damaged by acute injury or chronic degeneration is unable to be restored by an intrinsic healing process [1, 2]. This lack of self-repair may be related in part to the lack of a sufficient cell population to mediate the repair process. Cartilage has a low cellularity, with the chondrocyte population comprising less than 10% of the tissue volume, and chondrocyte proliferation and turnover in normal adult cartilage is limited. Moreover, chondrocytes are confined within a dense extracellular matrix, restricting cellular access to sites of damage. Mature articular cartilage also appears to lack a resident chondroprogenitor or stem cell population that could support tissue regeneration.

Articular cartilage is an avascular tissue, thereby negating access to potential progenitor cell populations from other sites. Structural damage that extends to the subchondral bone and causes bleeding in the damaged tissue results in the formation of a repair tissue. However, the repair tissue is most frequently a type of fibrocartilage, rather

than hyaline cartilage, and is unable to withstand repeated mechanical loading associated with normal joint motion due to its inferior mechanical properties, eventually resulting in tissue degeneration [68]. Repair techniques including drilling and abrasion purposely injure the underyling subchondral bone in order to induce bleeding and ultimately support fibrocartilage formation [2], which in certain situations may be an acceptable clinical outcome.

Clinical strategies for cartilage repair may involve the transfer of cells or tissue to the damaged site to promote healing. Transplantation of autologous chondrocytes [69, 70] and transplantation of osteochondral grafts [71-73], either autografts or allografts, are two approaches that have been used for treatment of focal defects and demonstrated good clinical outcomes. A pair of recent clinical reports provided direct comparisons of these two approaches and came to opposite conclusions regarding which was the better approach [74, 75]. Transplantation of soft-tissue grafts containing a chondroprogenitor population, such as periosteum or perichondrium, have been explored for repair of both focal and larger cartilage regions [76-78]. Currently for patients with extensive tissue loss, the primary treatment option beyond pain management strategies is total knee replacement surgery.

Cartilage Tissue Engineering: Strategies for *De Novo* Cartilage Development

During the last decade, much research has focused on the tissue engineering of articular cartilage as an alternative approach to current clinical options for cartilage repair. The primary objective of the tissue engineering approach is to promote complete regeneration of normal articular cartilage, and if necessary, the underlying subchondral bone. Guiding this objective is the belief that normal tissue function must be restored to

achieve long-term stability and clinical success. A major strategy for tissue engineering of articular cartilage has focused on growing *de novo* cartilage *in vitro*, with the viable cartilage tissue subsequently used to replace a region of damaged cartilage tissue *in vivo*. *De novo* cartilage has been produced *in vitro* through the combination of an appropriate cell population with a 3-D scaffold followed by sustained growth in serum-containing media.

The most frequently used cell source for tissue engineering approaches is the articular chondrocyte population. Upon isolation from native tissue, the articular chondrocyte population typically must be subjected to *in vitro* expansion to generate sufficient cell numbers for scaffold seeding. It has been demonstrated that monolayer expansion of articular chondrocytes can lead to dedifferentiation, or the loss of phenotype, in the expanded cell population, but recovery of phenotype is associated with reintroduction of the cells into a 3-D culture environment [37, 79, 80]. Problems associated with use of articular chondrocytes include: limited availability of appropriate cartilage tissue harvest sites, as these sites must be non-weight bearing; tissue donor site morbidity, as these harvest sites will not heal; increased patient cost and risk, as the tissue biopsy requires a surgical procedure; and limited expansion potential of the donor population [6, 9].

More recently, mesenchymal progenitor cell populations (MPCs) from tissues including periosteum, bone marrow, and adipose tissue have been shown to contain cells capable of chondrogenic differentiation when exposed to appropriate chemical factors [10-14]. Progenitor cells can be more easily harvested, are isolated from tissue that can be replenished or replaced, and have a higher proliferative potential as compared to articular chondrocytes [1]. Another advantage of using a progenitor cell source is that the

undifferentiated cells can be guided into cellular lineages for both cartilage and bone, thereby enabling development of complex tissue engineering scaffolds conducive for repair of an osteochondral defect [81, 82]. The use of progenitor cells in cartilage tissue engineering will need to be supported by optimization of *in vitro* conditions to stimulate proliferation and chondrogenic differentiation of progenitor cells in appropriate scaffolds.

The scaffold plays a central role in the development of *de novo* cartilage by providing an initial 3-D framework for cellular distribution and matrix accumulation. The most extensively studied scaffold materials shown to support *in vitro* tissue development have been the biodegradable synthetic polymers PGA, PLLA, and the copolymer PLGA [8, 83-85]. Gel-forming biomaterials have been utilized for chondrocyte encapsulation and have been demonstrated to support maintenance of the chondrocyte phenotype. These materials include fibrillar proteins, such as type I collagen [86, 87], naturally-occurring polysaccharides, such as agarose and alginate [36, 88, 89], and synthetic hydrogels, including PEG-based and peptide-based formulations [90, 91]. However, long-term studies demonstrating extensive *de novo* cartilage growth are lacking for most of these materials. A few approaches have evaluated developing *de novo* cartilage tissue in the absence of a scaffold material [92, 93], although producing a tissue of suitable dimensions could be a major obstacle.

The *in vitro* culture environment in which chondrocyte-seeded scaffolds are grown is another factor that contributes to *de novo* tissue development. Growth of scaffolds in a "static" environment, comparable to the method used for monolayer cell expansion in culture flasks, has been shown to produce a cartilage tissue that is lacking suitable tissue organization [68, 94]. Culture systems that incorporate media mixing or controlled media

flow, often termed bioreactors, provide several advantages over static culture including enhanced mass transport, a more controlled biochemical environment, and the application of biomechanical stimuli. A number of bioreactors have been designed to control the movement of culture media through and around the cell-seeded scaffolds, including perfusion bioreactors [95, 96], a rotating wall bioreactor [94], and a concentric cylinder bioreactor [97]. Culture systems have also been developed which apply mechanical stimulation to the scaffolds either through application of direct compressive deformation or through application of hydrostatic pressurization [15, 98, 99]. Growth factor supplementation, such as IGF-I or TGF-β1, has been combined with mechanical stimulation or bioreactor culture to further enhance matrix deposition and improve mechanical properties of the developing tissue [100, 101]. The most successful culture strategies have produced *de novo* cartilage after 4-8 weeks of growth having a glycosaminoglycan composition ~75% of native tissue, having a collagen composition ~10-40% of native tissue, and having mechanical properties ~20% of values measured in native tissue, with notably greater enhancement observed for selected mechanical parameters [102, 103].

Bone Marrow-Derived Adult Mesenchymal Progenitor Cells

Bone marrow contains cells of the hematopoietic lineage, in various stages of differentiation, and the nonhematopoietic cells that constitute the bone marrow stroma, including endothelial cells, adipocytes, and fibroblasts. Early evidence for the presence of skeletal progenitor cells in bone marrow was provided by diffusion chamber studies, which demonstrated *in vivo* bone and cartilage formation within diffusion chambers containing cells from bone marrow [104]. Since the diffusion chamber system allowed

nutrient exchange but blocked cellular interactions with the host environment, the new tissue directly resulted from the implanted cells. Subsequently, protocols for human bone marrow were developed to enable isolation of these progenitors, now commonly referred to as MPCs, based primarily on culture adherence and expansion on tissue culture plastic [105-107]. Similar protocols have been utilized for MPC isolation in multiple donor species including rat, rabbit, canine, equine, bovine, and goat [29, 106, 108-111].

MPCs, also called mesenchymal stem cells, have been postulated to have the potential to give rise to cell lineages which form all mesenchymal tissues [112]. Specific chemical factors have been shown to promote *in vitro* differentiation of MPCs into lineages for osteoblasts, chondrocytes, adipocytes, and marrow stromal cells [11, 113-116]. There has been recent evidence to suggest the possible existence of multiple stem cell populations in the bone marrow. A progenitor cell population, termed multipotent adult progenitor cells, has been isolated from bone marrow utilizing a low-serum or serum-free culture media combined with extensive monolayer cell expansion [117, 118]. This stem cell population has been shown to undergo *in vitro* and *in vivo* differentiation into cells from all three developmental lineages, including osteoblasts, endothelial cells, and neurons [119, 120].

The estimated frequency of MPCs in marrow is 1 in 100,000 nucleated cells [114, 121]. While the number of MPCs that can be isolated from a single marrow aspirate is relatively small, MPCs are capable of extensive self-renewal [122]. Thus, serial passaging can be used to notably expand the undifferentiated MPC population. In general, the ability of MPCs to differentiate upon exposure to appropriate signals is retained with passaging. Limited evidence suggests that the differentiation potential for

the chondrogenic lineage is sensitive to extensive passaging, but use of FGF-2 during monolayer expansion may help enhance this potential [123]. Aging has been shown to reduce the frequency of MPCs with osteogenic potential isolated from human and rodent donors [124-126]. Common orthopaedic diseases also appear to influence MPC characteristics. Decreased osteogenic potential was noted in MPCs from osteoporotic patients [127, 128]. MPCs taken from patients with advanced osteoarthritis were shown to have a reduced proliferative capacity and reduced chondrogenic and adipogenic potential, although osteogenic potential was normal [129].

The current, standard MPC isolation protocol is fairly simplistic, relying on adherent culture with growth in the presence of an optimized serum lot to select the MPC population [130]. Clonal analyses demonstrating osteogenic, chondrogenic, and adipodenic differentiation of progeny from a single MPC have provided direct evidence for the existence of a multilineage progenitor or stem cell. The observations in these studies that many clones supported only osteogenic differentiation may indicate that the isolated MPC population is a mixture of both stem cells and osteoprogenitors [113, 131]. A number of protocols utilizing specific cell-surface markers coupled with various cell-sorting techniques have recently been proposed for isolation of a better characterized MPC population [132-134].

MPCs are a promising cell source for regeneration and repair of orthopaedic tissues, such as cartilage and bone. *In vivo* osteochondrogenesis has been demonstrated for human, rat, and canine MPC donors [109, 135, 136]. These *in vivo* studies evaluated ectopic tissue formation in subcutaneous implants of cell-seeded ceramics or in peritoneal implants of cell-seeded diffusion chambers. One such study confirmed that new bone

formation was derived from the implanted MPCs rather than from host cells using immunocytochemical analyses [105]. Implanted MPCs have also contributed to cartilage and bone repair. Rabbit MPCs in collagen gels supported orthotopic tissue formation in osteochondral defects [108]. Canine MPCs seeded in ceramic carriers promoted healing of a segmental bone defect. While these studies involved the implantation of undifferentiated MPCs, more recent work has evaluated cartilage and bone formation resulting from MPCs that have been subjected to a specific culture medium supporting predifferentiation of the MPCs toward the chondrogenic or the osteogenic lineage prior to implantation [81, 82]. Notably, a two-week preculture of MPCs in chondrogenic supplements supported enhanced bone and cartilage formation over the implantation of unstimulated MPCs in subcutaneous implants of a hyaluronan-gelatin composite sponge [137].

Role of the Biomechanical Environment in Orthopaedic Tissue Development and Repair

The importance of the biomechanical environment in the growth and maintenance of bone and cartilage is well-established [58, 138, 139]. The biomechanical environment is also thought to play a role in skeletal tissue development and repair, mediated in part through influences on lineage commitment of stem cells and progenitor cell differentiation. *In vivo* observations have provided evidence for links between mechanical loading and skeletal tissue development and repair. Paralysis of chick embryos leads to reduced long bone growth [140, 141], which may be due to a disruption in loading of appendicular skeletal elements resulting from muscular contractions [18]. The degree of mechanical stability alters tissue development during fracture repair, suggesting that the local mechanical environment directly influences progenitor cell

differentiation at the fracture site [142, 143]. Rigid stabilization supports intramembranous bone formation, in which new bone forms directly from osteogenic differentiation of progenitor cells. Fixation that allows moderate interfragmentary movement in the fracture site supports endochondral bone formation, in which bone formation is preceded by cartilage formation and mineralization. During the endochondral process, new tissue formation involves successive waves of progenitor cell differentiation, first down the chondrogenic pathway and later down the osteogenic pathway.

In vitro studies have also provided evidence for loading influences on developmental regulation. Intermittent hydrostatic pressure applied to organ cultures of fetal long bone rudiments increased mineralized bone formation, which could be due in part to enhanced osteogenesis in these cultures [144]. Enhanced expression of markers for chondrogenesis were observed in chick limb bud cultures subjected to intermittent cyclic compressive loading, but not static loading, [19] and in mouse limb bud cultures subjected to constant static compression [20]. Additionally, a theory has been developed which describes how differences in local tissue mechanical stresses guide skeletal tissue development, growth, maintenance, and repair [18].

Based upon these observations from normal skeletal development and repair, mechanical stimulation may be an important factor influencing MPC differentiation and subsequent tissue development. A number of recent *in vitro* studies have evaluated mechanical loading influences on MPCs and other progenitor populations. Human marrow stromal cells subjected to oscillatory fluid flow exhibited increased proliferation and increased osteopontin and osteocalcin mRNA levels, markers of the osteoblast

phenotype [145]. Bovine bone marrow stromal cells cultured in chondrogenic media demonstrated increased gene expression for the chondrogenic markers aggrecan and type II collagen upon exposure to hydrostatic pressurization, but application of pressure alone was not sufficient to initiate differentiation [146]. Cyclic uniaxial strain transiently increased the expression of smooth muscle α-actin and smooth muscle-22α in MPCs after 1 day, while equiaxial strain downregulated expression of the smooth muscle cell markers [147]. Application of both rotational and translational strains, in the absence of specific chemical factors, led to the expression of collagen types I and III and tenascin-C, markers of ligament fibroblasts [148]. The results also showed evidence for ligament tissue development, including cellular alignment and formation of oriented collagen fibers, in response to mechanical stimulation for 21 days. Overall, these studies provide evidence that specific mechanical signals can direct or support differentiation of progenitor populations.

CHAPTER 3

TGF-β1 AND DEXAMETHASONE INFLUENCES ON *IN VITRO* GOAT MPC CHONDROGENIC DIFFERENTIATION AND MATRIX ACCUMULATION

Introduction

Mesenchymal progenitor cells (MPCs) are a promising cell source for orthopaedic tissue engineering therapies. MPCs associated with a carrier matrix have been shown to support new tissue formation in repair models for bone, cartilage, meniscus, and tendon [108, 149-151]. *In vitro* manipulations of MPCs have been utilized to initiate cellular differentiation and to promote development of tissue-engineered implants [82, 110]. In response to specific chemical cues, MPCs have been shown to undergo *in vitro* commitment to multiple mesenchymal cell lineages, including osteoblasts, chondrocytes, and adipocytes [113].

In vitro chondrogenic differentiation stimulated by growth factor supplementation has been demonstrated in marrow-derived MPCs for human, rabbit, equine, porcine, bovine, and goat donors [11, 12, 29, 110, 111, 152, 153]. *In vitro* chondrogenesis has been supported in a variety of culture systems. These include scaffold-free cultures such as high-density monolayers and micromass pellets [11, 153, 154], biomatrices such as alginate, gelatin, and hyaluronan [38, 155, 156], and polymeric scaffolds such as PGA, PLLA, and PLGA/PEG [25, 110, 157]. The majority of *in vitro* studies have been performed with human MPCs, in part to demonstrate the feasibility of developing either cell transplantation or tissue engineering strategies for cartilage repair utilizing MPCs. Ultimately, the development of cartilage repair strategies based upon MPCs will include preclinical testing in appropriate cartilage defect models. Preclinical testing will be

supported by *in vitro* validation of chondrogenic differentiation in nonhuman MPC sources and by evaluation of differentiating MPCs in 3-D culture systems appropriate for cartilage tissue engineering applications.

This chapter explores TGF-β1 and dexamethasone (Dex) influences on *in vitro* goat MPC chondrogenic differentiation and subsequent matrix accumulation in alginate suspension. This work was guided by established human MPC protocols, which routinely used a combination of TGF-β (isoforms 1, 2, or 3) and Dex in ITS^{+}-containing media to promote chondrogenic differentiation of MPCs in various 3-D culture systems [11, 12, 25]. One objective of this work was to define culture media conditions that supported MPC chondrogenic differentiation and that enhanced matrix accumulation by differentiating MPCs in hydrogel suspension. The number of supplements provided (single versus multiple supplements) and the duration over which supplements were provided (early versus entire culture period) were variables evaluated in defining the culture media conditions. Criteria for comparison of culture media conditions included the ability to support production of cartilage matrix components and the amount of matrix accumulation.

A second objective of this work was to establish a time course for matrix synthesis and accumulation by MPCs undergoing chondrogenic differentiation and subsequently expressing a chondrogenic phenotype. A four-week study was therefore conducted utilizing the defined culture media conditions identified in the first objective. Multiple MPC donors were evaluated, in part to provide an assessment of donor variability. Overall, the studies conducted in this chapter provided knowledge about goat MPCs

undergoing chondrogenic differentiation in alginate suspension, which would be important to planning of future loading studies.

The evaluation of chondrogenic differentiation utilized biochemical analyses and histological analyses to validate the production of the two major structural components of articular cartilage: proteoglycans and type II collagen. Proteoglycan production was assessed quantitatively by determining proteoglycan synthesis, measured as the ^{35}S-sulfate incorporation rate, and sulfated glycosaminoglycan (sGAG) accumulation. Type II collagen production was assessed qualitatively with immunohistochemical staining. Protein synthesis, measured as the ^{3}H-proline incorporation rate, and DNA content were also evaluated as general culture markers.

Methods

Materials

Goat MPCs were provided by Osiris Therapeutics, Baltimore, MD. All media, trypsin, PBS, culture additives (unless noted otherwise), and pepsin were from Invitrogen Life Technologies, Chicago, IL. FBS was from Hyclone, Logan, UT. Ascorbic acid-2-phosphate, L-proline, papain, 1,9-dimethyl-methylene blue (DMMB), chondroitin sulfate A, and buffer reagents for the biochemical analyses were from Sigma-Aldrich, St. Louis, MO. ITS^{+} premix was from BD Biosciences, Bedford, MA. Recombinant human TGF-β1 and recombinant human FGF basic were from R&D Systems, Minneapolis, MN. Pronova UP LVG alginate was from NovaMatrix, FMC BioPolymer, Norway. L-5-^{3}H-proline was from Amersham Biosciences, Piscataway, NJ. ^{35}S-sodium sulfate and Ecolume were from MP Biomedical, Irvine, CA. The Live-Dead kit and the PicoGreen

kit were from Molecular Probes, Eugene, OR. Immunohistochemistry reagents were from Vector Labs, Burlingame, CA.

MPC Isolation and Expansion

Osiris Therapeutics conducted the goat MPC isolation from iliac crest bone marrow aspirates followed by adherent primary expansion as described in their published methods for human MPCs [38]. It should be noted that goat MPC donors provided for use in these studies had been subjected to a screening process to verify their ability to undergo chondrogenic differentiation (personal communication with Osiris Therapeutics). Following primary culture, the cells were expanded for two passages (plating density 2700cells/cm^2) in low glucose DMEM with 110mg/L sodium pyruvate and 584ml/L glutamine containing 10% FBS, 1% antibiotic/antimycotic **(**100units/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericinB), and 1ng/ml FGF-2. The use of FGF-2 was based upon previous work with canine MPCs (see Appendix A) and upon other studies in the literature demonstrating a positive effect for FGF-2 on maintenance of MPC differentiation potential following monolayer expansion [123, 158]. When cells had reached a near-confluent density, the flasks were incubated with 0.42g/L trypsin, 0.44mM EDTA·4Na in Ca^{2+}-free, Mg^{2+}-free PBS followed by rinsing in standard expansion media to yield a single cell suspension.

3-D MPC Culture

Passage 2 MPCs were suspended in one part Ca^{2+}-free, Mg^{2+}-free PBS and mixed with 2 parts 3% Pronova UP LVG alginate (prepared in Ca^{2+}-free, Mg^{2+}-free PBS and filter sterilized) to yield a cell suspension of $12.5x10^6$cells/ml. The 2% alginate concentration, somewhat higher than the 1.2% concentration used previously with MPCs, was consistent

with alginate formulations used in mechanical loading studies with chondrocytes. The seeding concentration used was determined empirically by selecting a concentration that was an intermediate value for the range of concentrations used previously for hydrogel suspension of chondrocytes and MPCs. The cell suspension (25-45μl depending on desired thickness) was transferred to individual 4mm diameter wells of a custom-designed casting mold (Figure 1). The casting mold consisted of four pieces (as assembled from bottom to top): solid polycarbonate base, wire mesh, cellulose acetate membrane filter (0.2 micron pore size), and a top polycarbonate piece with a 4x4 grid of 4mm diameter holes drilled out. The filter was wet with the polymerization solution (102mM $CaCl_2$, 10mM Hepes, 100mM NaCl) prior to addition of the cell suspension. After ~25 minutes of incubation, polymerization solution was added to the top of the wells. Gels were removed from the wells after a total incubation of ~60 minutes and incubated in 150mM NaCl for ~40 minutes, followed by incubation in chondrogenic media excluding the ascorbic acid-2-phosphate, Dex, and TGF-β1 (see below) for ~40 minutes. Gels were then transferred to 48-well plates and cultured throughout the study in chondrogenic media: high glucose DMEM with 110mg/L sodium pyruvate and 584ml/L glutamine containing 1% ITS^+ premix (6.25μg/ml insulin, 6.25μg/ml transferrin, 6.25ng/ml selenious acid, 1.25mg/ml bovine serum albumin, 5.35mg/ml linoleic acid), 1% antibiotic-antimycotic, 40μg/ml L-proline, and 50μg/ml ascorbic acid-2-phosphate. Chondrogenic supplements evaluated were 100nM Dex and 10ng/ml TGF-β1 as per previous protocols [12, 25]. Cultures received fresh media changes every 1-2 days.

Experimental Design

TGF-β1 and Dex effects on chondrogenic differentiation and subsequent matrix accumulation were explored in a series of four studies (Table 1). At the designated harvest time in all studies, gels were either subjected to biochemical or histological analyses.

Study 1: Media Supplement Comparison

Media formulations were compared with regard to the ability to support MPC chondrogenic differentiation. Media lacking supplements was compared to media continuously supplemented with Dex only, with TGF-β1 only, and with both Dex and TGF-β1. Gels were cultured for 14 days (2 donors; 1 experiment/donor).

Study 2: Duration of Dex Supplementation

Since the first study indicated that Dex had a negative effect on the progression of chondrogenic differentiation for TGF-β1 supplemented cultures, a study was subsequently conducted to more thoroughly examine whether some combination of Dex and TGF-β1 supplementation would support progression of chondrogenic differentiation in a manner comparable to that supported by TGF-β1 alone. In this study, the duration of Dex supplementation was varied, while TGF-β1 supplementation was continuous. Gels were initially cultured with TGF-β1 or with Dex and TGF-β1. Dex was removed from the supplemented media at a designated culture time (day 2, day 4, or day 7) and all gels were grown to day 14 (1 donor; 1 experiment).

Study 3: Duration of TGF-β1 Supplementation

Since the first study demonstrated that TGF-β1 was stimulatory for MPC chondrogenic differentiation, a study was subsequently conducted to determine if the stimulatory action

of TGF-β1 was dependent on continuous supplementation, thereby providing greater understanding about TGF-β1 influences on MPC cultures. In this study, removing TGF-β1 at either culture day 4 or day 7 was compared to continuous TGF-β1 supplementation in day 14 cultures (1 donor; 1 experiment).

Study 4: Time Course of MPC Matrix Synthesis and Accumulation

In the preceding studies, chondrogenic differentiation and matrix accumulation were analyzed at culture day 14. However, preparation for the loading studies necessitated understanding how synthesis and accumulation of cartilage matrix components changed with time for MPCs cultured with chondrogenic media. A study was thus conducted evaluating MPC/alginate cultures subjected to continuous TGF-β1 supplementation and grown to 28 days, with samples harvested at multiple time points (2 donors; 1 experiment/donor).

Radiolabel Incorporation

At harvest, samples for biochemical analyses (n=3-4 per condition per donor) were transferred to 96 well plates and incubated for 20 hours in 0.5ml culture media additionally containing 20μCi/ml L-5-^{3}H-proline and 10μCi/ml ^{35}S-sodium sulfate. At the end of the incubation period, the samples were transferred to new culture wells containing 1 ml of radiolabel quench solution, which was PBS additionally containing 0.8mM sodium sulfate and 1.0mM L-proline. Samples were washed in a total of four fresh aliquots of quench solution for ~30 minutes per wash. Samples were then transferred to preweighed tubes, weighed, and stored at -20ºC until further processing.

Biochemical Analyses

Gels were lyophilized, weighed, resuspended in papain digest solution (150mM sodium phosphate dibasic, 5mM Na_2EDTA, 5mM n-acetyl-L-cysteine pH 6.5 containing ~1.7 units papain per sample), and incubated at 60ºC for 24 hours. For radiolabel incorporation measurements, 100µl of the sample digest was added to 2 ml Ecolume in a scintillation vial. Raw counts in CPM were obtained for each sample using a liquid scintillation counter. ^{3}H and ^{35}S activity were calculated from the raw counts based upon standard curves prepared from serial dilutions of the original radiolabeled media. The activities were then converted into ^{3}H-proline and ^{35}S-sulfate incorporation rates. Double-stranded DNA content was determined using the PicoGreen assay and quantified against a lambda DNA standard curve. A 1:20 sample dilution was prepared in the base digest buffer. 100µl sample dilution was mixed with 100µl PicoGreen reagent (prepared in 1X TE buffer according to the vendor instructions). The mixture was incubated for 10 minutes and then measured on a Perkin-Elmer HTS 7000plus plate reader (excitation at 485nm; emission at 535nm). sGAG content was determined using a modified DMMB assay appropriate for use with alginate-containing samples [159]. sGAG content in experimental samples was quantified against a chondroitin-4-sulfate standard from bovine cartilage. Alginate, at a concentration matching that in the sample dilutions, was included in the buffer used to create the chondroitin-4-sulfate standard curve to account for any interaction between the dye and alginate. Sample dilutions were prepared as necessary in the base digest buffer. 200µl dye (~21mg/L dye in 29mM sodium formate, pH 1.5 adjusted with formic acid) was added to 20µl sample dilution in a 96-well plate and then measured immediately at 525nm on a Bio-Tek PowerWave X340 plate reader.

Cell Viability Staining

In selected studies control gels were rinsed in PBS three times for 10 minutes per rinse, halved, stained with a Live-Dead kit utilizing 4μM calcein and 4μM ethidium homodimer, and subsequently rinsed in PBS three times for 10 minutes per rinse. To assess cellular viability at the designated culture time, the stained gels were imaged on a Zeiss LSM 510 confocal microscope.

Histological Analyses

At harvest, samples for histological analyses (n=1-2 per condition per donor) were halved. Gel halves were rinsed in PBS, soaked in 100mM $BaCl_2$ for ~45 minutes to stabilize crosslinking of the alginate gel, rinsed in PBS, transferred to 10% neutral-buffered formalin for 36-48 hours, rinsed in PBS and stored in 70% alcohol. Samples were subsequently embedded in paraffin and sectioned at 5μm for analysis. Immunohistochemical assays were performed (see Appendix B for protocol) with a monoclonal antibody provided by the Developmental Studies Hybridoma Bank for Type II collagen (IgG II-II6B3). Following deparaffinization and rehydration, tissue sections were incubated in pepsin for ten minutes and the incubation was repeated a second time. Presence of the antigen was detected as an alkaline phosphatase deposit. To control for nonspecific staining, one slide per batch was incubated with blocking serum rather than the primary antibody and then processed identically with the other slides. Positive and negative control slides were also included.

Statistical Analyses

Data were analyzed using a general linear ANOVA model with Tukey's test for post-hoc analysis (significance at $p<0.05$), where the effect of media condition (or culture day for

the time course study) on the biochemical parameter of interest was evaluated for a given MPC donor in each study.

Results

Media Supplement Comparison

Continuous supplementation with Dex or TGF-β1 was compared to growth in baseline media containing ascorbic acid-2-phosphate (AA2P) for day 14 MPC cultures. Chondrogenic differentiation was supported by TGF-β1 supplementation, but was not supported by Dex supplementation or by the AA2P baseline media (Table 2, data shown for donor G115). Chondrogenic differentiation was demonstrated by a 20-fold increase in ^{35}S-sulfate incorporation from day 1 to day 14, by sGAG/DNA accumulation, and by staining for type II collagen (Figure 2). The inclusion of TGF-β1 in the culture media also promoted cellular proliferation, as DNA content increased 30% ($p<0.001$) from day 1 to day 14. Conversely, DNA content decreased from Day 1 to Day 14 in both the Dex and AA2P groups (67% and 72% respectively; $p<0.001$). Live/Dead staining demonstrated that cell viability was initially high in the day 1 cultures, while at day 14 a majority of cells were viable in the TGF-β1 cultures, a minority of cells were viable in the Dex cultures, and very few cells were viable in the AA2P cultures (Figure 3).

Continuous supplementation with TGF-β1 was further compared to continuous supplementation with both Dex and TGF-β1 (D&T) for day 14 MPC cultures. Matrix synthesis and accumulation were markedly reduced in the Dex-containing media (Figure 4). The addition of Dex to media supplemented with TGF-β1 decreased ^{35}S-sulfate incorporation (85% for donor G115; 66% for donor G151; $p<0.001$) and similarly

decreased sGAG/DNA (89% for donor G115; 71% for donor G151; $p<0.001$) compared to culture in TGF-β1 alone. There were no significant differences in DNA content between the D&T and TGF-β1 groups (data not shown). Positive staining for type II collagen was present around a majority of cells for the TGF-β1 supplemented media but was present around only a very limited number of cells for the D&T supplemented media (Figure 2), indicating that the MPC/alginate cultures exposed to both Dex and TGF-β1 had progressed more slowly in the differentiation process than cultures exposed to TGF-β1 alone by culture day 14.

Duration of Dex Supplementation

The initial study indicated that Dex had a negative effect on the progression of chondrogenic differentiation for TGF-β1 supplemented cultures. However, the dose of Dex, which would depend on both supplement concentration and duration, may have influenced the response of the MPC cultures. The duration of Dex supplementation was subsequently evaluated in combination with continuous TGF-β1 supplementation to determine if some combination of Dex and TGF-β1 could support progression of chondrogenic differentiation in a manner comparable to that supported by TGF-β1 alone and/or could be beneficial for MPC matrix synthesis and accumulation in alginate suspension as compared to levels promoted by TGF-β1 alone. Removal of Dex from the culture media after two, four, or seven days of culture (i.e. Dex 0-2, Dex 0-4, Dex 0-7) resulted in ^{35}S-sulfate incorporation and DNA levels that were comparable to the No Dex (i.e. TGF-β1 only) group at day 14 (Figure 5; DNA data not shown). In contrast, sGAG/DNA was more sensitive to Dex duration. Both the Dex 0-4 and the Dex 0-7 groups had lower sGAG/DNA levels compared to the No Dex group (17% and 50%

decrease respectively; $p<0.02$), while sGAG/DNA for the Dex 0-2 group was comparable to the No Dex group. Consistent with the previous study, the cultures that received Dex throughout the study (Dex 0-14) had 69% lower ^{35}S-sulfate incorporation ($p<0.001$) and 75% lower sGAG/DNA ($p<0.001$) compared to the No Dex group, while DNA content was comparable across the two groups (data not shown). Positive staining for type II collagen was present around a majority of cells in the No Dex gels, the Dex 0-2 gels, and the Dex 0-4 gels (Figure 5). In contrast, a minority of cells exhibited positive staining in the Dex 0-7 gels, while only a very limited number of cells exhibited positive staining in the Dex 0-14 gels. The type II collagen staining indicated that the negative effect of Dex on the progression of chondrogenic differentiation in response to TGF-β1 required the extended presence of Dex in the culture media. However, shorter periods of Dex supplementation in combination with continuous TGF-β1 supplementation were not found to have a positive effect on either ^{35}S-sulfate incorporation or sGAG/DNA content as compared to TGF-β1 alone. Since there was no compelling reason to use Dex, even for a short duration, to support either MPC chondrogenic differentiation or matrix accumulation, Dex was excluded from the culture media in all subsequent studies.

Duration of TGF-β1 Supplementation

While the initial study demonstrated that TGF-β1 was stimulatory for MPC chondrogenic differentiation, it was unknown if continuous TGF-β1 supplementation was necessary as compared to a shortened duration of TGF-β1 supplementation. Furthermore, it was uknown if sustained TGF-β1 supplementation in a serum-free culture media was beneficial for MPC matrix synthesis and accumulation in hydrogel suspension. The duration of TGF-β1 supplementation was subsequently evaluated.

TGF-β1 was included throughout the 14 day culture period (TGF 0-14) or was removed from the media beginning on Day 4 (TGF 0-4) or Day 7 (TGF 0-7). To demonstrate changes in time with supplementation, baseline gels were analyzed at day 4. Comparing the day 14 culture groups (Table 3) to the day 4 baseline showed that ^{35}S-sulfate incorporation had approximately doubled at day 14 from the day 4 level of 0.1034±0.0073 pmol/ngDNA/hr and sGAG content had increased in the range of 23- to 28-fold at day 14 from the day 4 level of 8.0±1.4 μg/gel for the range of TGF-β1 supplement durations evaluated. These increases, together with the detection of type II collagen staining in all day 14 cultures (not shown), indicated that the progression of chondrogenic differentiation from day 4 to day 14 was maintained in MPC cultures for all durations of TGF-β1 supplementation evaluated. However, differences in matrix synthesis and accumulation were noted between the day 14 culture groups. TGF-β1 supplementation beyond the initial 4 days in culture led to an increase in ^{35}S-sulfate incorporation (11% for TGF 0-7; 13% for TGF 0-14; $p<0.02$) and sGAG content (16% for TGF 0-7; 21% for TGF 0-14; $p<0.05$) compared to the TGF 0-4 group, while both parameters were comparable between the TGF 0-7 and TGF 0-14 groups. The extended use of TGF-β1 also increased the DNA content compared to the TGF 0-4 group (34% for TGF 0-7; 73% for TGF 0-14; $p<0.002$) and compared to the TGF 0-7 group (29% for TGF 0-14; $p<0.001$). Therefore, per cell sGAG accumulation (sGAG/DNA) was increased in the TGF 0-4 group compared to the other groups (15% versus TGF 0-7; 42% versus TGF 0-14; $p<0.02$) and in the TGF 0-7 group compared to the TGF 0-14 group (24%; $p<0.003$). This study demonstrated that an initial 4 day period of TGF-β1 supplementation was a sufficient stimulus for chondrogenic differentiation. However,

continuous TGF-β1 supplementation was chosen as the chondrogenic stimulus for subsequent studies due to the noted enhancements in ^{35}S-sulfate incorporation and sGAG content corresponding to extended TGF-β1 supplementation.

Time Course of MPC Matrix Synthesis and Accumulation

In the preceding studies, chondrogenic differentiation and matrix accumulation were evaluated at culture day 14. However, it was planned that the loading studies would compare differentiating MPC cultures demonstrating different levels of matrix production, requiring an understanding of how matrix synthesis and accumulation changed with time in culture. Therefore, a study was conducted to evaluate matrix synthesis and accumulation for MPC/alginate cultures continuously supplemented with TGF-β1 over a four-week period (Figures 6 and 7, data shown for donors G115 and G117). Cartilage matrix components appeared during the first week of culture. Gels at culture day 1 exhibited low ^{35}S-sulfate incorporation and were lacking both sGAG/DNA accumulation and type II collagen staining. With additional culture to day 4, gels exhibited intermediate ^{35}S-sulfate incorporation and low sGAG/DNA accumulation, but were lacking type II collagen staining. With further culture to day 7, gels exhibited peak or near-peak ^{35}S-sulfate incorporation (4.8-fold and 2.7-fold increase from day 4 to day 7 for donors G115 and G117 respectively; $p<0.001$), increasing sGAG/DNA accumulation (8.6-fold and 6.7-fold increase from day 4 to day 7 for donors G115 and G117 respectively; $p<0.02$), and a low level of type II collagen staining. Comparing the time course for individual donors indicated strong similarities between them during the first week of culture.

Further changes in matrix synthesis and accumulation occurred from day 7 to day 28 and some differences were noted between the two donors. For donor G115, ^{35}S-sulfate incorporation further increased 29% from day 7 to day 14 ($p<0.001$) and then decreased 10% from day 14 to day 28 ($p<0.003$). For donor G117, ^{35}S-sulfate incorporation decreased 18% from day 7 to 14 ($p<0.001$) and then further decreased 34% from day 14 to day 28 ($p<0.001$). Despite the differences in ^{35}S-sulfate incorporation, sGAG/DNA increased steadily from day 7 to day 28 in both donors. DNA content increased from day 7 to day 14 (48% for donor G115; 59% for donor G117; $p<0.001$), then decreased 10% ($p<0.02$) for donor G115 from day 14 to day 28 but did not change for donor G117. ^{3}H-proline incorporation decreased from day 7 to day 14 (29% for donor G115; 44% for donor G117; $p<0.001$), then increased from day 14 to day 28 (120% for donor G115; 47% for donor G117; $p<0.001$). Type II collagen was detected surrounding a limited number of cells at day 7 and a majority of cells at day 14, with pericellular staining increasing through day 28 (Figure 7).

Discussion

Marrow-derived MPCs are a promising cell source for cartilage tissue engineering strategies and much research has focused on characterizing *in vitro* chondrogenic differentiation of human MPCs. However, preclinical testing of cartilage repair strategies utilizing MPCs necessitates the establishment *in vitro* culture systems promoting chondrogenic differentiation of nonhuman MPCs. Goats have been used increasingly for cartilage repair studies in recent years. While previous studies have demonstrated *in vitro* chondrogenic differentiation of goat MPCs [29, 30], knowledge about the effects of specific media components on differentiation is limited. This chapter evaluated *in vitro*

goat MPC chondrogenic differentiation and subsequent matrix accumulation in alginate suspension in response to TGF-β1 and Dex supplementation.

This work provided biochemical and immunohistochemical evidence that goat MPC/alginate cultures exposed to TGF-β1 supplementation undergo chondrogenic differentiation. TGF-β1 was found to be sufficient to support differentiation, without additional supplementation with Dex. This result is in contrast to prior work with human MPC pellet cultures, which were shown to require both TGF-β and Dex for differentiation [12], but is consistent with prior work in rabbit MPC pellet cultures [153]. Previous *in vitro* studies by other groups typically evaluated MPC chondrogenic differentiation in response to continuous growth factor and glucocorticoid supplementation. However, one group reported that human MPCs seeded in polylactide/alginate amalgam underwent chondrogenic differentiation with exposure to 50 ng/ml TGF-β1 for the initial three days of culture, a dose of TGF-β1 five times higher than the concentration used for continuous supplementation [25]. Our results indicated that continuous supplementation with 10ng/ml TGF-β1 was not a requirement for differentiation of goat MPCs. Rather, commitment to the chondrogenic lineage in response to TGF-β1 supplementation appeared to occur rapidly in the goat MPC/alginate cultures, as progression of chondrogenic differentiation evidenced by extensive type II collagen staining in day 14 cultures was maintained following TGF-β1 removal after 4 or 7 days in culture.

The primary role of TGF-β1 in MPC chondrogenesis is consistent with previous studies in other undifferentiated cell populations. TGF-β1 and other members of the TGF-β family have promoted *in vitro* chondrogenesis in a variety of progenitor cell

populations, including limb bud cultures, mesenchymal cell lines, and periosteal explants [54, 56, 57, 160-164]. While the effects of TGF-β1 on chondrogenic differentiation are well established, the mechanisms that underlie these effects are only beginning to be explored. The expression of TGF-β receptors by human MPCs has been verified [39, 165]. Recent reports have demonstrated the importance of MAP kinase signaling cascades and Wnt signaling pathways in mediating the actions of TGF-β1 on MPC chondrogenic differentiation [166, 167].

The sustained presence of TGF-β1 in the culture media could mediate changes in the MPC cultures beyond those overtly related to differentiation. TGF-β1 has been shown to influence proliferation and extracellular matrix synthesis in numerous cell types [168-172]. More specifically related to cartilage, TGF-β1 has been demonstrated to support proliferation of both MPCs [110, 173, 174] and articular chondrocytes [175-177]. Our work showed that TGF-β1 supported proliferation of goat MPCs in alginate suspension. The influence of TGF-β1 on matrix production by primary articular chondrocytes has been variable [178]. In some cultures matrix production was stimulated and in other cultures production was inhibited with exposure to TGF-β1 [179-182]. Our work demonstrated a stimulation of proteoglycan synthesis in day 14 MPC/alginate cultures for continuous TGF-β1 supplementation as compared to supplementation only during the initial four days in culture. Overall, the evaluation of TGF-β1 effects on matrix production in differentiating MPC cultures was very limited in our work and focused on varying the duration of supplementatation at a single concentration (10ng/ml). However, the concentration of TGF-β1 most appropriate for initiating chondrogenic differentiation in MPC cultures could be different from the concentration most appropriate for

stimulating matrix production in committed MPC cultures. Furthermore, once the MPCs have become differentiated chondrocytes, other growth factors shown previously to stimulate matrix production by primary chondrocytes, such as IGF-I [101], could be more benefical than TGF-β1 for extended MPC culture. Although these considerations were beyond the scope of the thesis work, such issues could be critical to the *in vitro* development of tissue-engineered cartilage constructs utilizing MPCs.

While the TGF-β1 effects on goat MPCs were consistent with previous studies, the Dex effects on goat MPCs were notably different. To our knowledge, our work is the first to report a negative influence for Dex on TGF-β1 stimulated chondrogenic differentiation of marrow-derived MPCs. Continuous Dex supplementation slowed, but did not completely disrupt, differentiation of the goat MPC/alginate cultures. Extended culture in Dex and TGF-β1 may have supported further progression of differentiation of the MPC cultures. A recent study reported chondrogenic differentiation of goat MPC/hydrogel cultures after six weeks of supplementation with Dex and TGF-β1 [183]. Our work also demonstrated that Dex supplementation, when shortened to the initial two to four days of culture, supported chondrogenic differentiation and subsequent matrix accumulation similarly to culture in the absence of Dex for MPCs continuously supplemented with TGF-β1. Although altering the Dex concentration from the standard concentration of 100nM used in this work and previous studies could possibly support enhanced matrix production by differentiating MPCs, the levels of matrix synthesis and accumulation supported by TGF-β1 alone were considered to be sufficient for the purposes of the thesis work and the Dex issue was not explored further.

The culmination of the results from the studies evaluating TGF-β1 and Dex effects demonstrated that continuous TGF-β1 supplementation in media lacking Dex was the best culture media for goat MPC chondrogenic differentiation and matrix accumulation based upon the supplement levels and durations evaluated. This culture media was shown to support the appearance of cartilage matrix components during the first week of culture. Culture from day 7 to day 14 supported further progression of chondrogenic differentiation as related to type II collagen expression, while culture beyond day 14 contributed to matrix accumulation. The timing study further demonstrated that distinctions could be made between MPC cultures at days 1, 4, and 7 as related to proteoglycan synthesis, sGAG/DNA accumulation, and type II collagen expression. Given the limited exposure to TGF-β1 supplementation, the day 1 culture could be described as a prechondrogenic culture. The increase in proteoglycan synthesis and the detection of sGAG/DNA accumulation at day 4 represented changes by a differentiating culture, while the appearance of type II collagen at culture day 7 indicated a further progression of chondrogenic differentiation. Although MPCs at these three culture times have unique biosynthetic profiles, it is unknown whether MPCs at these different culture times are distinct in other ways, such as their ability to respond to a variety of external factors, including mechanical stimuli.

In conclusion, TGF-β1 was demonstrated to be a potent stimulator of chondrogenic differentiation in goat MPC/alginate cultures. The chondrogenic response to TGF-β1 was notably enhanced by the exclusion of Dex, a factor used routinely with MPCs from other species. Importantly, the work conducted in this chapter provided the experimental foundation upon which the remaining thesis work was built. Having established an

appropriate culture media to promote chondrogenic differentiation and having gained a basic understanding regarding the progression of differentiation and matrix accumulation in the alginate culture system, explorations into how differentiating MPC cultures might be modulated by specific environmental factors could begin.

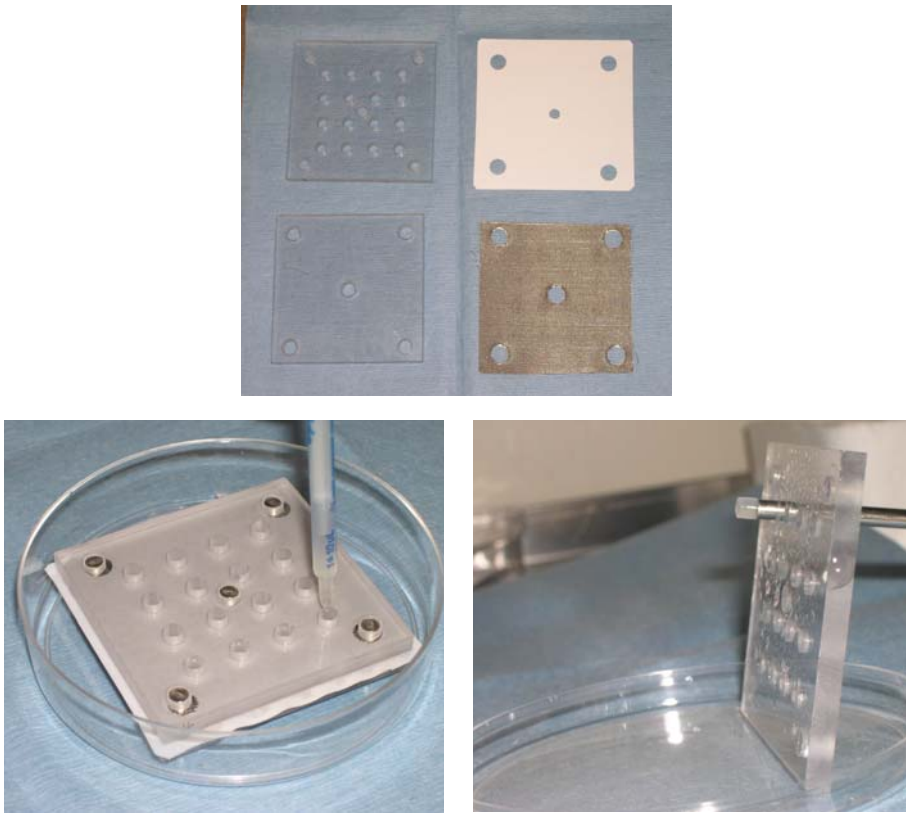


Figure 1: Gel Casting Mold. Top: The mold consists of a top piece with 4mm diameter holes drilled out, a cellulose acetate membrane filter, a wire mesh, and a solid base piece (shown clockwise from upper left) and is held together by screws. Bottom left: Cell/alginate suspension is pipetted into the wells of the assembled mold after the filter is wet with a polymerization solution. Bottom right: After polymerization, the mold is disassembled and the cylindrical gels are removed from the wells by pushing a rod through the bottom of each well.

Table 1: Summary of Studies Evaluating TGF-β1 and Dex Effects. In the first 3 studies, cultures were harvested for comparison on day 14 only.

	Experimental Variable	Levels Evaluated
Study 1	Media Supplement	Dex; TGF-β1; Dex & TGF-β1; Comparison to baseline media with ascorbic acid-2-phosphate
Study 2	Days of Dex Supplementation *Note: All cultures received TGF-β1*	Dex starting on day 0 for 0, 2, 4, 7, or 14 days
Study 3	Days of TGF-β1 Supplementation	TGF-β1 starting on day 0 for 4, 7, or 14 days
Study 4	Days in Culture *Note: All cultures received TGF-β1*	1, 4, 7, 14, and 28 days

Table 2: Day 14 Evaluation of Media Supplements for Chondrogenic Differentiation Potential. Continuous supplementation with Dex or TGF-β1 was compared to growth in baseline media containing ascorbic acid-2-phosphate (AA2P). Baseline data for day 1 cultures are provided to demonstrate changes in time with supplementation. (mean ± SEM; n=4gels/supplement; data shown for donor G115). Undetectable indicates that the sGAG level was below the lowest chondroitin sulfate standard of the DMMB assay.

Supplement	DNA (ng)	sGAG (μg)	sGAG/DNA (μg/μg)	^{35}S-Sulfate Incorporation (pmol/ngDNA/hr)
AA2P	1005 ± 12	Undetectable	Undetectable	0.0027 ± 0.0001
Dex	1198 ± 9	Undetectable	Undetectable	0.0103 ± 0.0007
TGF-β1	4730 ± 37	219.7 ± 11.3	46.4 ± 2.2	0.3475 ± 0.0096
Day 1 Cultures	3624 ± 78	Undetectable	Undetectable	0.0176 ± 0.0008

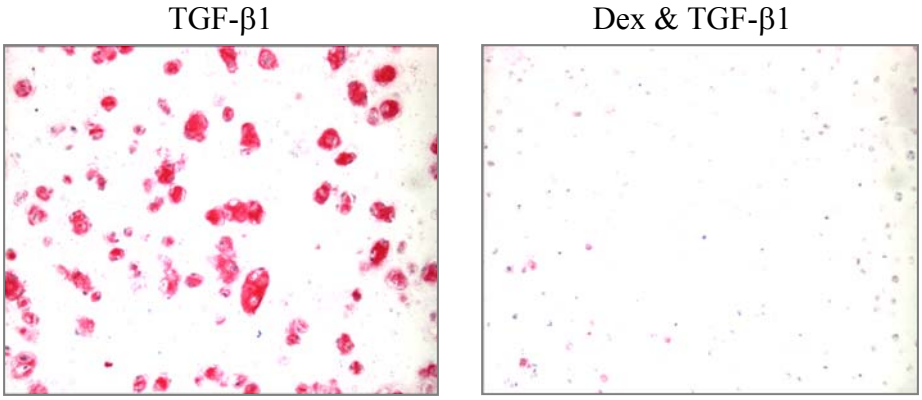


Figure 2: Type II Collagen Staining of Day 14 Cultures Comparing Chondrogenic Media Conditions. Type II collagen immunostaining (20X; red coloration indicates presence of antigen; images shown for donor G115) for cultures supplemented with TGF-β1 only or with Dex & TGF-β1.

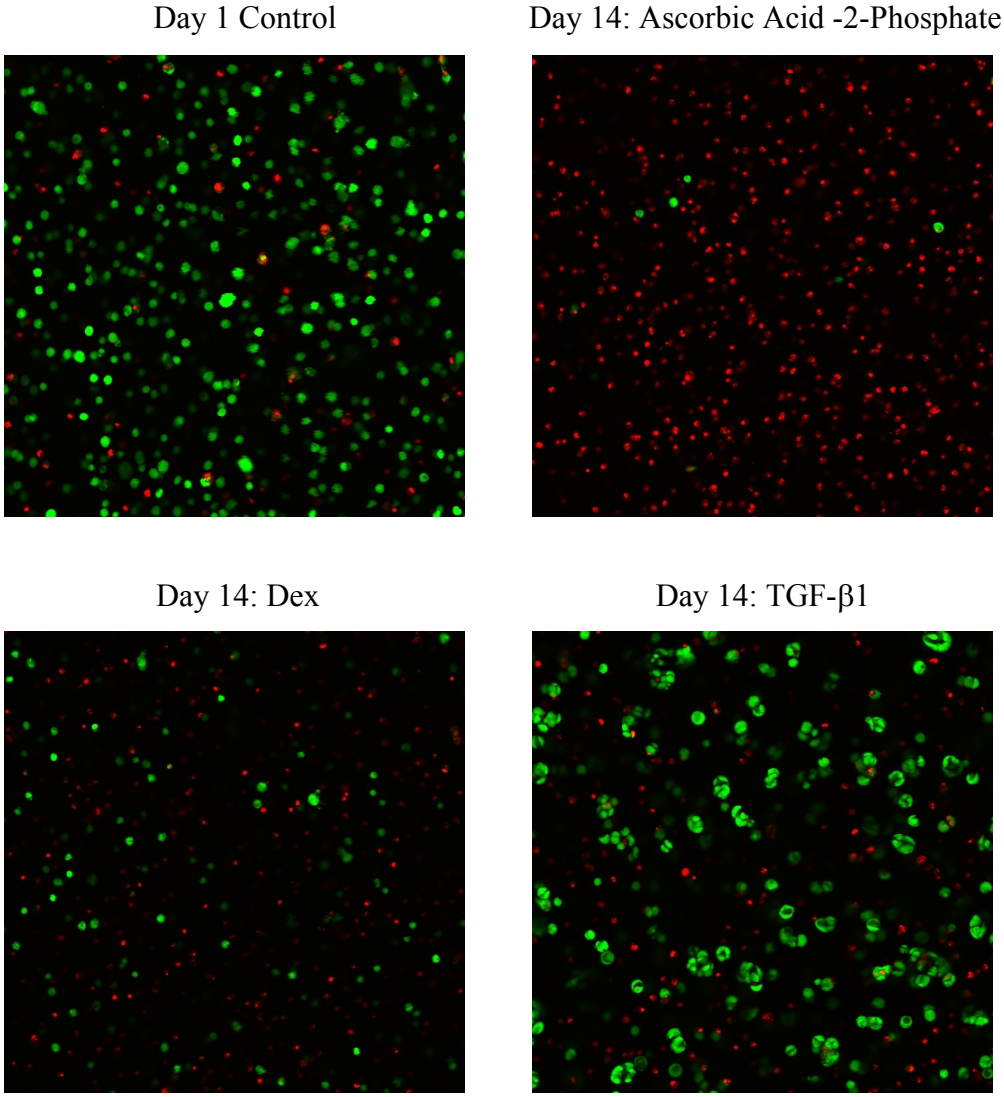


Figure 3: Cell Viability Staining of Cultures Grown in Different Media Conditions. Live/Dead staining (10X; images shown for donor G115) comparing viability in Day 1 control cultures to Day 14 cultures grown in baseline media with ascorbic acid-2-phosphate, in media with Dex, or in media with TGF-β1. Green staining (calcein AM) indicates a viable cell, while red staining (ethidium homodimer) indicates a dead cell.

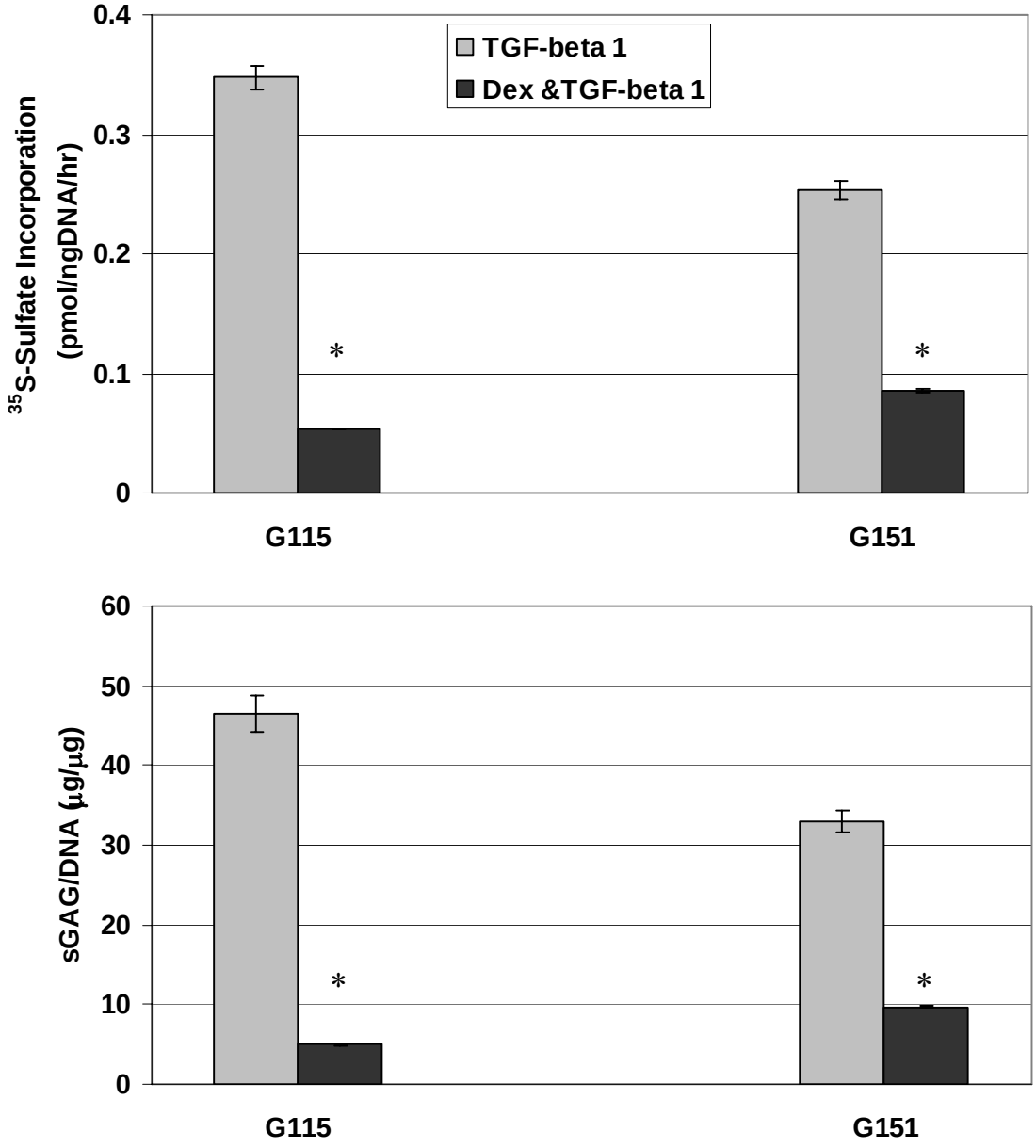


Figure 4: Biochemical Analyses Comparing Chondrogenic Media Conditions. Cultures were supplemented with TGF-β1 only or with Dex & TGF-β1 in two MPC donors. Bars represent mean ± SEM (n=4gels/donor/supplement; 1 experiment). * denotes significantly different versus TGF-β1 only for the designated donor.

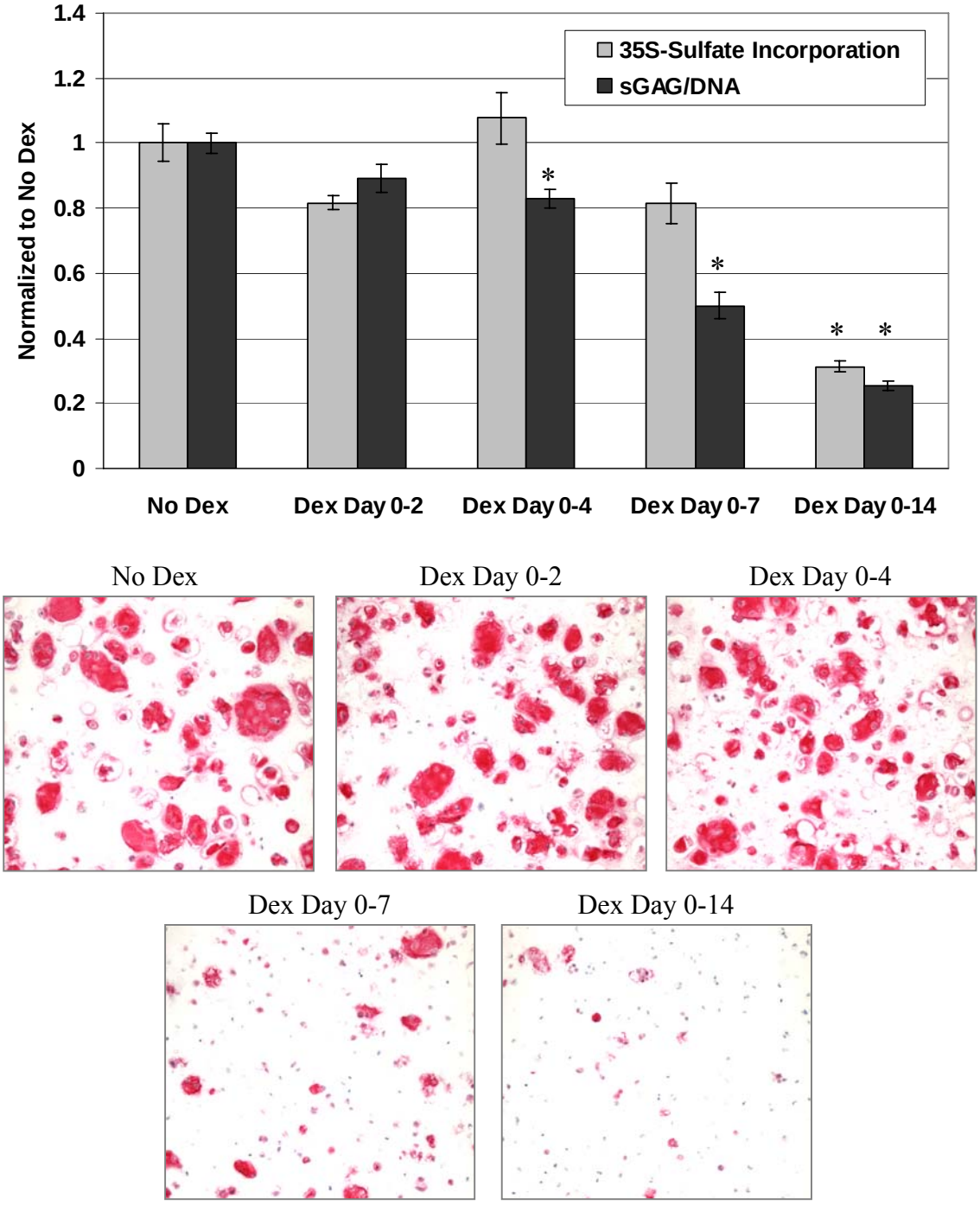


Figure 5: Evaluation of Day 14 Cultures in Response to Varying the Duration of Dex Supplementation. Dex was excluded from the media (No Dex), was included for the initial 2, 4, or 7 days of culture, or was included throughout the culture period (Dex Day 0-14). All cultures were supplemented with TGF-β1. Top: Evaluation of ^{35}S-sulfate incorporation and sGAG/DNA. Bars represent mean ± SEM (n=4gels/supplement; 1 experiment). * denotes significantly different versus No Dex. Bottom: Type II collagen immunostaining (20X; red coloration indicates presence of antigen).

Table 3: Evaluation of Day 14 Cultures in Response to Varying the Duration of TGF-β1 Supplementation. TGF-β1 was included throughout the culture period (0-14) or was removed from the media beginning on day 4 (0-4) or day 7 (0-7). Values are mean ± SEM (n=4gels/supplement; 1 experiment). * denotes significantly different versus TGFβ-1 0-14.

Supplement	DNA (ng)	sGAG (μg)	sGAG/DNA (μg/μg)	^{35}S-Sulfate Incorporation (pmol/ngDNA/hr)
TGF-β1 0-14	6155 ± 196	220.0 ± 3.0	35.8 ± 0.8	0.2313 ± 0.0056
TGF-β1 0-7	* 4768 ± 213	211.2 ± 10.3	* 44.4 ± 1.8	0.2275 ± 0.0046
TGF-β1 0-4	* 3560 ± 60	* 181.3 ± 6.5	* 50.9 ± 1.0	* 0.2043 ± 0.0033

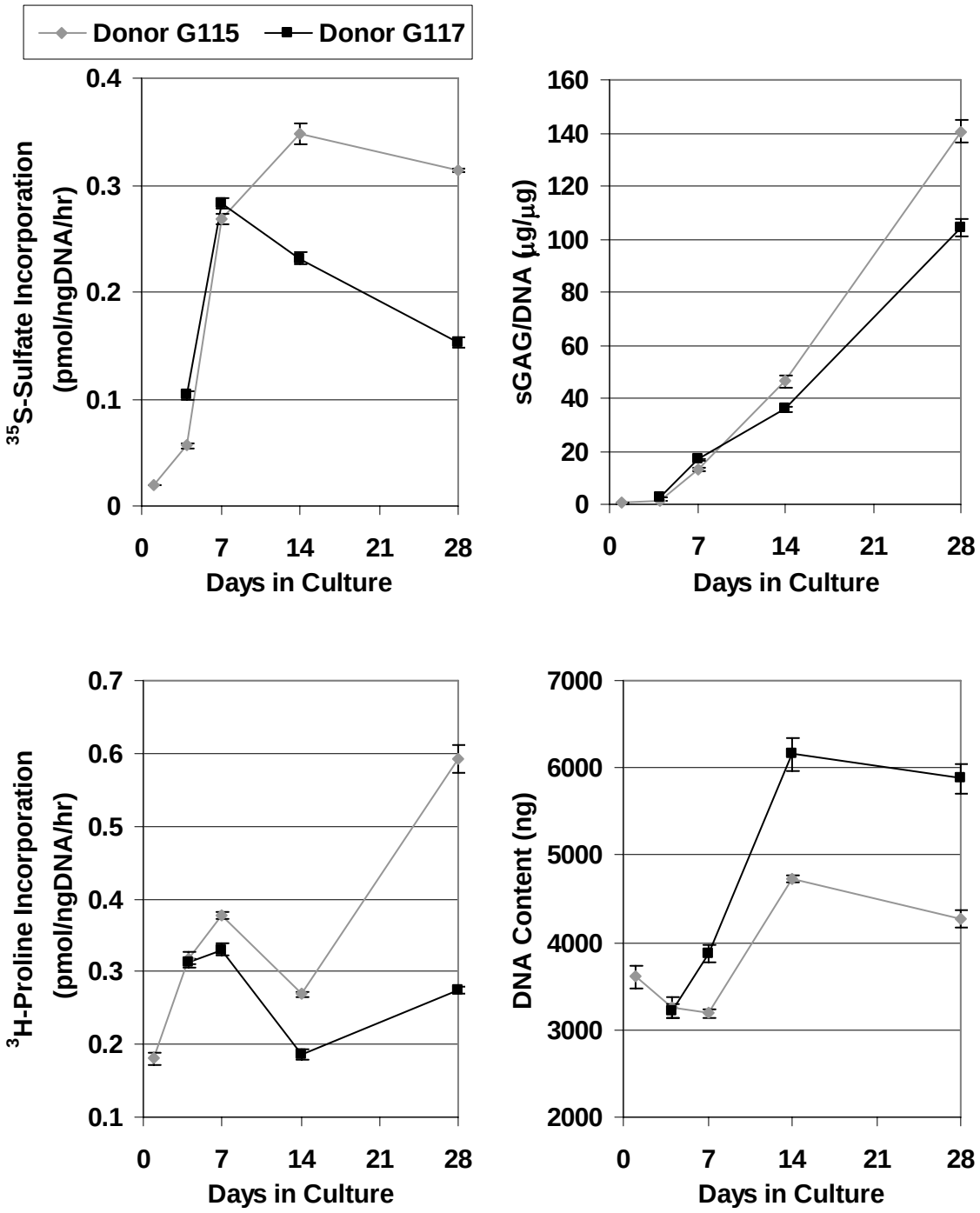


Figure 6: Time Course of Biosynthesis and Matrix Accumulation for MPC Cultures undergoing Chondrogenic Differentiation. Cultures were grown from 1 day to 4 weeks in media supplemented with TGF-β1 for two MPC donors (G115 and G117). Note that day 1 cultures were excluded from the experiment for donor G117. Values are mean ± SEM (n=4gels/culture time/donor; 1 experiment).

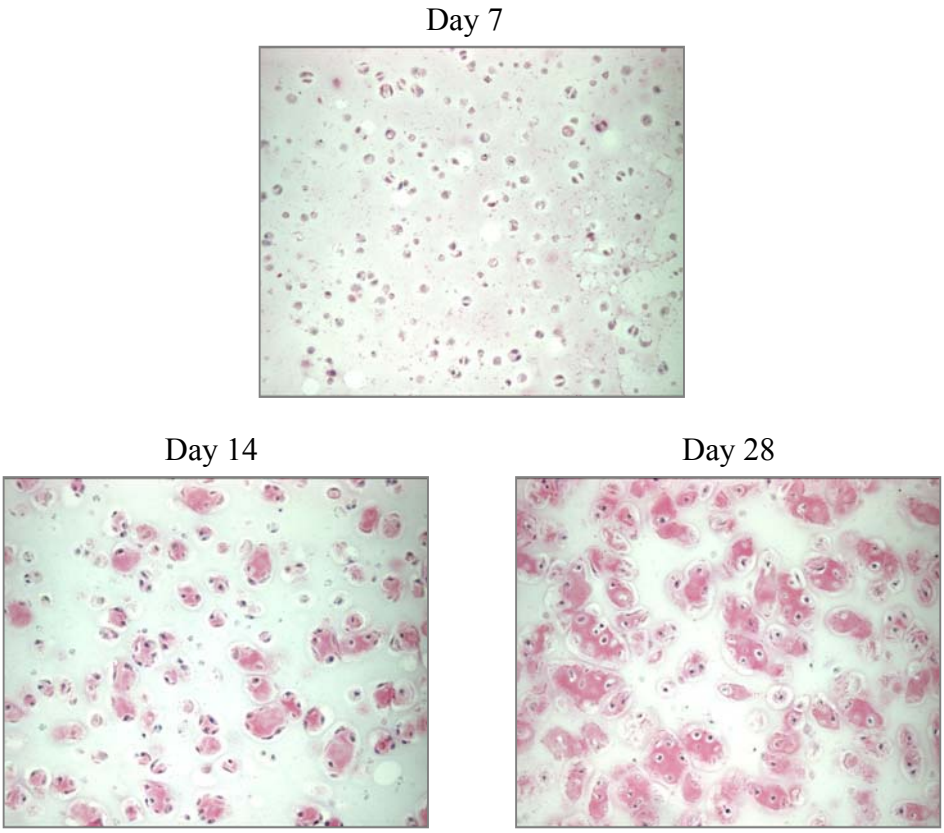


Figure 7: Time Course of Type II Collagen Staining in Chondrogenic Cultures. Type II collagen immunostaining (20X; red coloration indicates presence of antigen; shown for donor G117) for cultures supplemented with TGF-β1 and grown to days 7, 14, and 28.

CHAPTER 4

OSCILLATORY COMPRESSIVE LOADING EFFECTS ON MPC CHONDROGENIC ACTIVITY

Introduction

Recent strategies for cartilage tissue engineering have utilized adult mesenchymal progenitor cells (MPCs) in combination with chondrogenic growth factors and an appropriate scaffold to support *in vitro* cellular differentiation and tissue development [137, 155, 184]. Mechanical preconditioning has been proposed as a stimulatory factor for the development of tissue-engineered cartilage constructs and articular chondrocytes have been shown to produce an enhanced cartilage matrix when subjected to long-term oscillatory compressive loading [15-17]. However, little is known about the ability of mechanical signals to influence MPC chondrogenic differentiation and matrix accumulation *in vitro*.

A growing body of evidence indicates that mechanical signals direct *in vivo* development and repair of orthopaedic tissues, which may be mediated in part by influences on lineage commitment and progression of mesenchymal progenitor populations. In prenatal development, muscular contractions provide loading of appendicular skeletal elements [18]. This stimulation may be critical in guiding limb expansion, as paralysis of chick embryos leads to reduced long bone growth [140, 141]. Tissue development during postnatal fracture repair is influenced by the degree of mechanical stability at the fracture site. Rigid stabilization supports direct new bone formation, while fixation that allows moderate interfragmentary movement in the fracture site promotes cartilage formation prior to new bone formation [142, 143]. Mechanical

stimulation has also been shown to support cartilage development by postnatal progenitor populations. Periosteal autografts that were placed free-floating into the knee joint were found to form cartilage to an increased degree with the application of continuous passive motion compared to grafts subjected to joint immobilization [185].

Experimental *in vitro* evidence for mechanical loading influences on chondrogenesis is limited. Constant static compression of mouse limb bud cultures in gel suspension resulted in enhanced gene expression of the chondrogenic markers type II collagen and aggrecan [20]. Cyclic compressive loading increased ^{35}S-sulfate incorporation and matrix staining in chick limb bud suspension cultures, while static loading elicited no effect [19]. Dynamic fluid pressure, when applied at low levels, enhanced proteoglycan matrix staining and type II collagen accumulation in rabbit periosteal explants suspended in agarose [186]. A study with human MPCs cultured as scaffold-free pellets under conditions promoting chondrogenic differentiation found that the application of cyclic hydrostatic pressure early in the culture period resulted in increased proteoglycan and collagen contents at 2-4 weeks of culture [187]. While this study provided evidence that differentiating MPCs are able to sense mechanical signals in an *in vitro* setting, chondrogenic differentiation and tissue development in the pellet culture format has limited relevance for cartilage tissue engineering considerations. Studies evaluating mechanical loading influences on MPCs in a 3-D culture system that is compatible with development of a tissue-engineered cartilage construct are needed.

Mechanical loading could be beneficial to multiple stages of the *in vitro* development of tissue-engineered cartilage constructs, including MPC chondrogenic differentiation, matrix production by differentiating and maturing cultures, and long-term tissue growth.

This chapter describes the evaluation of oscillatory compressive loading effects on the chondrogenic activity of MPC cultures suspended in alginate, where chondrogenic activity is an indicator of matrix production by differentiating cultures. The effects of oscillatory mechanical stimulation on differentiating MPC cultures were evaluated by assessing the chondrogenic activity following application of daily 3 hour loading periods for 3 consecutive days. Since the duration of exposure to chondrogenic media preceding loading initiation could modulate the MPC response to mechanical loading, oscillatory unconfined compressive loading was initiated following a variable preculture period of 1 to 21 days in media containing TGF-β1, a supplement demonstrated to support MPC chondrogenic differentiation. It was hypothesized that dynamic compressive loading in combination with chondrogenic supplements would enhance the chondrogenic activity of MPCs relative to the influence of supplements alone in a preculture time-dependent manner. Proteoglycan synthesis, measured as the ^{35}S-sulfate incorporation rate, and proteoglycan accumulation, measured as sulfated glycosaminoglycan (sGAG) accumulation, were utilized to assess MPC chondrogenic activity and were supported by the detection of type II collagen expression. Protein synthesis, measured as the ^{3}H-proline incorporation rate, and DNA content were also evaluated as general culture markers.

Methods

Materials

Goat MPCs were provided by Osiris Therapeutics, Baltimore, MD. Bovine stifle joints were from Research 87, Marlborough. MA. All media, trypsin, PBS, culture additives (unless noted otherwise), and pepsin were from Invitrogen Life Technologies, Chicago,

IL. FBS was from Hyclone, Logan, UT. Ascorbic acid-2-phosphate, L-proline, agarose, papain, DMMB, chondroitin sulfate A, and buffer reagents for the biochemical analyses were from Sigma-Aldrich, St. Louis, MO. Agarase was from Calbiochem, EMD Biosciences, San Diego, CA. ITS^+ premix was from BD Biosciences, Bedford, MA. Recombinant human TGF-β1 and recombinant human FGF basic were from R&D Systems, Minneapolis, MN. Pronova UP LVG alginate was from NovaMatrix, FMC BioPolymer, Norway. Type II collagenase was from Worthington Biochemicals, Lakewood, NJ. L-5-^{3}H-proline was from Amersham Biosciences, Piscataway, NJ. ^{35}S-sodium sulfate and Ecolume were from MP Biomedical, Irvine, CA. The PicoGreen kit was from Molecular Probes, Eugene, OR. Immunohistochemistry reagents were from Vector Labs, Burlingame, CA.

MPC Isolation and Expansion

Osiris Therapeutics conducted the goat MPC isolation from iliac crest bone marrow aspirates followed by adherent primary expansion as described in their published methods for human MPCs [38]. Following primary culture, the cells were expanded for two passages (plating density 2700cells/cm^2) in low glucose DMEM with 110mg/L sodium pyruvate and 584ml/L glutamine containing 10% FBS, 1% antibiotic/antimycotic **(**100units/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericinB), and 1ng/ml FGF-2. When cells had reached a near-confluent density, the flasks were incubated with 0.42g/L trypsin, 0.44mM EDTA·4Na in Ca^{2+}-free, Mg^{2+}-free PBS followed by rinsing in standard expansion media to yield a single cell suspension.

3-D MPC Culture

Passage 2 MPCs were suspended in one part Ca^{2+}-free, Mg^{2+}-free PBS and mixed with 2 parts 3% Pronova UP LVG alginate (prepared in Ca^{2+}-free, Mg^{2+}-free PBS and filter sterilized) to yield a cell suspension of $12.5x10^6$cells/ml. 45µl of the cell/alginate suspension was transferred to individual 4mm diameter wells of a custom-designed casting mold. The cellulose acetate membrane filter positioned beneath the wells was wet with the polymerization solution (102mM $CaCl_2$, 10mM Hepes, 100mM NaCl) prior to addition of the cell suspension. After ~25 minutes of incubation, polymerization solution was added to the top of the wells. Cylindrical gels were removed from the wells after a total incubation of ~60 minutes and incubated in 150mM NaCl for ~40 minutes, followed by incubation in chondrogenic media excluding the ascorbic acid-2-phosphate and TGF-β1 (see below) for ~40 minutes. In one experiment, agarose gels were also cast by combining an equal volume of cells in media with 4% agarose (type VII low-gelling agarose powder in Ca^{2+}-free, Mg^{2+}-free PBS; autoclaved to sterilize) to yield a 2% agarose solution containing $12.5x10^6$cells/ml. 45µl of the cell/agarose suspension to the wells of the casting mold, with filter paper prewet with PBS positioned beneath. The agarose cultures gelled upon cooling to room temperature in the culture hood and were subsequently extracted from the molds and rinsed similarly to the alginate gels. All gels were transferred to 48-well plates and cultured throughout the study in chondrogenic media (unless noted otherwise): high glucose DMEM with 110mg/L sodium pyruvate and 584ml/L glutamine containing 1% ITS^+ premix (6.25µg/ml insulin, 6.25µg/ml transferrin, 6.25ng/ml selenious acid, 1.25mg/ml bovine serum albumin, 5.35mg/ml

linoleic acid), 1% antibiotic-antimycotic, 40μg/ml L-proline, 50μg/ml ascorbic acid-2-phosphate, and 10ng/ml TGF-β1. Cultures received fresh media changes every 1-2 days.

Compressive Loading

For loading initiation following the designated preculture period, gels were sized to 3mm thickness using a custom-designed sizing tool (Figure 8), transferred to bioreactors, and subjected to unconfined uniaxial compressive deformation within the confines of a standard tissue culture incubator (37ºC, 5%CO_2). The daily loading protocol consisted of applying a dynamic load (15±3% compression at 1 Hz; sinusoidal waveform) or a 15% static load (15% compression) for 3 hours, followed by a cessation of loading for 21 hours. Control gels were subjected to 0% static load for 3 hours. Gel cultures were subjected to 3 days of consecutive loading (or 6 days in selected experiments). With completion of the final loading day, gels were transferred to free swelling culture. Unless noted otherwise, MPC gels were grown in culture media containing TGF-β1 throughout the duration of each study.

There were no previous reports of compressive loading of MPCs in alginate suspension that could be used to guide the selection of loading parameters. Frequency, compression peak amplitude, and compression static offset levels were therefore based upon protocols utilized with MPCs and chondrocytes in agarose suspension [24, 99, 188, 189]. The selection of the 3 hour loading interval was based on previous studies with MPCs and other prechondrogenic cultures, where dynamic loading was applied for 1 to 4 hours per day for multiple consecutive days [19, 188, 190].

For dynamic loading, gel disks were subjected to oscillatory uniaxial unconfined compression using a compression device developed previously for studies with

chondrocyte cultures [191]. The compression device consisted of bioreactor cassettes, the loading frame, a digital control unit, and a power supply (Figure 9). Each polycarbonate bioreactor cassette had a base containing 8 individual cylindrical wells into which the gels were placed and had a top with a cylindrical, solid, stainless steel platen located above the position of each well. The cassette base was fixed to the stationary base plate of the loading frame and two cassettes could be positioned onto opposite sides of the loading frame. The position of the cassette top, and thus the amount of compressive deformation applied to the gels, was determined by the position of the upper plate of the loading frame to which the top was mounted, and this upper plate was capable of controlled, uniaxial motion. A linear stepper motor was attached to the base plate of the loading frame. The upper plate of the loading frame rode on the lead screw of the stepper motor and was stabilized by three vertical guide posts. Lead screw rotation was thus was converted into vertical motion of the upper plate. Lead screw rotation was confirmed with a rotary encoder. Digital control was used to apply an oscillatory motion profile. To approximate a sinusoidal waveform, the load cycle was discretized into a series of thirty step motions.

For static loading, gel disks were subjected to a static uniaxial unconfined compression using a polycarbonate bioreactor (Figure 9), in which gel disks were contained in individual wells in the base of the reactor and the lid of the bioreactor had individual solid platens that were located above each well. The position of the lid of the reactor, and thus the amount of compressive deformation applied to the gels, was determined by the height of machined spacer bars. The spacer bars fit into aligned grooves in the base and lid of the bioreactor. At the end of the 3-hour loading period, the

spacer bars were replaced by bars with a height of ~3.4mm **(**except for the initial G117 loading experiment in which replacement bars with a height of ~3.0mm were used). Prior to loading initiation on consecutive loading days, the dynamic and static bioreactors were disassembled, gels were repositioned within the reactor wells, the bioreactors were reassembled, and all gels were fed fresh media.

Experimental Design

The effects of oscillatory compressive loading on MPC chondrogenic activity were explored in a series of three studies (Table 4). At the designated harvest time in all studies, gels were either subjected to biochemical or histological analyses.

Study 1: Time-Dependent Loading Effects on MPC Chondrogenic Activity

Early differentiating cultures were the focus for exploring the interaction between the preculture period in chondrogenic media and the MPC response to oscillatory loading. In the initial loading experiment, MPCs were cultured for 1, 4, or 7 days and then loading was applied for 3 consecutive days. Gels were harvested after the final loading day or on day 14. This experiment was conducted at separate times in two different donors. A primary difference was observed between the responses of the two donors for the early (day 1-3) loading interval. Further clarification of the influence of early loading on MPCs was needed, so a portion of the initial loading experiment was repeated. MPCs were cultured for 1 day, loading was applied for 3 consecutive days, and gels were harvested on day 14. To more thoroughly evaluate the potential donor influence on the response to loading, the repeat experiment was conducted in four donors in parallel.

Study 2: Secondary Influences on the MPC Response to Dynamic Loading

Given the limited responsiveness of MPC cultures to oscillatory loading in the initial loading study, a second study was conducted to evaluate the contribution of selected experimental variables on the MPC response to dynamic loading. The study was divided into two experiments. The first experiment evaluated the impact of both longer-term stimulation and TGF-β1 supplementation in a single MPC donor. Gels were initially cultured with TGF-β1 supplementation to initiate chondrogenic differentiation. Concurrent with loading initiation on day 4, half of the gels were cultured in media lacking TGF-β1. Loading was applied for 6 consecutive days, rather than 3 days as used in previous experiments, and gels were harvested on day 14. The second experiment focused on the hydrogel material utilized in culturing the MPCs and compared compressive loading of MPCs in alginate and agarose gels for a single MPC donor. Loading was initiated on day 1 for 3 consecutive days and cultures were harvested on day 14.

Study 3: Comparing Loading Effects on MPC Chondrogenic Activity for Intermediate and Extended Preculture Times

The previous loading studies focused on differentiating MPC cultures and indicated a lack of modulation of chondrogenic activity in response to oscillatory compressive loading. However, a differentiated MPC culture with substantial matrix accumulation could have a different sensitivity to oscillatory loading effects on chondrogenic activity than the differentiating MPC cultures. A study was conducted to explore this issue. Loading effects on MPC chondrogenic activity were compared for loading initiated at day 7, an intermediate preculture time corresponding to a differentiating MPC culture, and for loading initiated at day 21, an extended preculture time corresponding to a

differentiated MPC culture. Loading was applied for 6 consecutive days and gels were harvested after the final loading day (1 donor; 1 experiment). Concurrent with loading initiation, half of the MPC gels were cultured in media lacking TGF-β1 to assess possible interactions between loading and TGF-β1 supplementation.

Radiolabel Incorporation

At harvest, samples for biochemical analyses (n=4-6) were transferred to 96 well plates and incubated for 20 hours in 0.5ml culture media additionally containing 20 μCi/ml L-5-^{3}H-proline and 10 μCi/ml ^{35}S-sodium sulfate. At the end of the incubation period, the samples were transferred to new culture wells containing 1 ml of radiolabel quench solution, which was PBS additionally containing 0.8mM sodium sulfate and 1.0mM L-proline. Samples were washed in a total of four fresh aliquots of quench solution for ~30 minutes per wash. Samples were then transferred to preweighed tubes, weighed, and stored at -20ºC until further processing.

Biochemical Analyses

Alginate gels were lyophilized, weighed, resuspended in papain digest solution (150mM sodium phosphate dibasic, 5mM Na_2EDTA, 5mM n-acetyl-L-cysteine pH 6.5 containing ~1.7 units papain per sample), and incubated at 60ºC for 24 hours. Agarose gel digestion involved a multi-step process. Lypophilized samples were weighed, resuspended in the base digest buffer, heated to 70ºC for 30 minutes, cooled to 40ºC, incubated for two hours after receiving a concentrated agarase solution (final concentration ~5 mUnits per sample), and then incubated overnight at 60º after receiving a concentrated papain solution (final concentration ~1.7 units per sample). For radiolabel incorporation measurements, 100μl of the sample digest was added to 2 ml Ecolume in a scintillation

vial. Raw counts in CPM were obtained for each sample using a liquid scintillation counter. ^{3}H and ^{35}S activity were calculated from the raw counts based upon standard curves prepared from serial dilutions of the original radiolabeled media. The activities were then converted into ^{3}H-proline and ^{35}S-sulfate incorporation rates. Double-stranded DNA content was determined using the PicoGreen assay and quantified against a lambda DNA standard curve. A 1:20 sample dilution was prepared in base buffer. 100µl sample dilution was mixed with 100µl PicoGreen reagent (prepared in 1X TE buffer according to the vendor instructions). The mixture was incubated for 10 minutes and then measured on a Perkin-Elmer HTS 7000plus plate reader (excitation at 485nm; emission at 535nm). sGAG content was determined using a modified DMMB assay appropriate for use with alginate-containing samples [159]. sGAG content in experimental samples was quantified against a chondroitin-4-sulfate standard from bovine cartilage. Alginate, at a concentration matching that in the sample dilutions, was included in the buffer used to create the chondroitin-4-sulfate standard curve to account for any interaction between the dye and alginate. Sample dilutions were prepared as necessary in the appropriate base digest buffer. 200µl dye (~21mg/L dye in 29mM sodium formate, pH 1.5 adjusted with formic acid) was added to 20µl sample dilution in a 96 well plate and then measured immediately at 525nm on a Bio-Tek PowerWave X340 plate reader.

Histological Analyses

At harvest, samples were halved (n=1-2). Gel halves were rinsed in PBS, soaked in 100mM $BaCl_2$ for ~45 minutes (alginate gels only), rinsed in PBS, transferred to 10% neutral-buffered formalin for 36-48 hours, rinsed in PBS and stored in 70% alcohol. Samples were subsequently embedded in paraffin and sectioned at five microns for

analysis. Immunohistochemical assays were performed (see Appendix B for protocol) with a monoclonal antibody provided by the Developmental Studies Hybridoma Bank for Type II collagen (IgG II-II6B3). Following deparaffinization and rehydration, tissue sections were incubated in pepsin 2 x 10 minutes. Presence of the antigen was detected as an alkaline phosphatase deposit. To control for nonspecific staining, one slide per batch was incubated with blocking serum rather than the primary antibody and then processed identically with the other slides. Positive and negative control slides were also included.

Statistical Analyses

Data were analyzed using a general linear ANOVA model with Tukey's test for post-hoc analysis (significance at $p<0.05$), where the effect of loading group on the biochemical parameter of interest was evaluated for a given MPC donor in each study. Depending on the study, the effect of loading group was evaluated with respect to the designated loading interval and harvest time, the designated gel material, or the designated media condition. While the data were presented normalized to the 15% static control group for each experiment, the raw data were used for statistical analyses.

Results

Time-Dependent Loading Effects on MPC Chondrogenic Activity

Based upon an MPC time course study conducted previously that demonstrated the appearance of cartilage matrix components by culture day 7 in response to TGF-β1 supplementation, the loading experiments were initiated during the first week of culture to focus on the earliest differentiating cultures. Culture days 1, 4, and 7 were used for loading initiation and loading was applied for a 3-day interval, thereby enabling

comparisons between differentiating MPC cultures that exhibited different levels of matrix production at the time that loading was initiated. In the initial loading experiment two culture endpoints were selected to allow the evaluation of both early (day after loading completion) and sustained (day 14) loading effects. The experiment was conducted first in donor G117 and then was repeated at a separate time in donor G115. In the G117 cultures the loaded day 4-6 samples were significantly damaged due to an uncontrolled movement of the loading frame, and it was therefore not possible to include these samples in the endpoint analyses.

For donor G117, the influence of dynamic loading depended on the timing of loading initiation (Figure 10, Figure 11). For loading initiated on day 1, there were no early loading effects. All loading groups were comparable at Day 4 for the parameters measured. However, with additional culture to day 14, chondrogenic activity of the MPCs was modulated by dynamic loading. The dynamic group had mean values for ^{35}S-sulfate incorporation (25% decrease versus 15% static group; 22% decrease versus 0% static group; $p<0.001$) and sGAG/DNA (27% decrease versus 15% static group; 23% decrease versus 0% static group; $p<0.001$) significantly lower than the static groups. In the day 14 gels, fewer cells stained for type II collagen in the dynamically loaded cultures as compared to the statically loaded cultures (Figure 12). At day 14, the dynamic group also had significantly lower DNA (29% decrease versus 15% static group; 21% decrease versus 0% static group; $p<0.01$) and significantly higher ^{3}H-proline incorporation (27% increase versus 15% static group; 16% increase versus 0% static group; $p<0.05$) compared to the static groups following loading from day 1-3. It should be noted that a deviation from the standard loading protocol occurred with the loaded day 1-3 samples.

The dynamically loaded gels were held under static compression during the 21 hour rest period on the first day of loading. On the two subsequent loading days, the loading platens were held at a vertical position greater than the height of the gels, comparable to what was done in all other loading intervals in the G117 experiment and in all future experiments.

For loading initiated on day 7 in the G117 cultures, chondrogenic activity was found to be comparable in all loading groups for each of the evaluation times (Figure 10, Figure 11; Type II collagen staining not shown). However, one notable transient effect of dynamic loading was detected. In day 10 cultures the dynamic group had significantly higher ^{35}S-sulfate incorporation compared to the static groups (13% increase versus 15% static group; 16% increase versus 0% static group; $p<0.005$), but in day 14 cultures ^{35}S-sulfate incorporation was comparable across all loading groups. The increase in ^{35}S-sulfate incorporation at day 10 was not associated with enhanced sGAG/DNA in the dynamic group as compared to the static groups at either day 10 or day 14. At day 10 the dynamic group also had significantly lower DNA content compared to the 0% static group (11%; $p<0.01$), but no other differences in DNA were observed at either time. ^{3}H-proline incorporation was comparable across all loading groups at days 10 and 14. Furthermore, the two static groups were comparable for all parameters measured for each loading interval and harvest time evaluated.

For donor G115, dynamic loading did not modulate the chondrogenic activity of the MPC cultures for any of the three loading initiation times. For each loading interval and harvest time evaluated, all loading groups were comparable for ^{35}S-sulfate incorporation, sGAG/DNA, and Type II collagen staining, as well as DNA content and ^{3}H-proline

incorporation, with one exception (Figure 13; Type II collagen staining not shown; DNA data not shown). For the day 4-6 loading interval, the dynamic group exhibited a decrease in both ^{35}S-sulfate incorporation (12%; $p<0.05$) and ^{3}H-proline incorporation (12%; $p<0.005$) compared to the 15% static group at day 7, but there were no differences between the dynamic group and the 0% static group at day 7 or between the dynamic and static groups at day 14 for either incorporation measurement.

In comparing the response of the two donors, a major difference was noted for the loaded day 1-3 set. Dynamic loading resulted in a decrease in the chondrogenic activity of day 14 cultures for donor G117 but did not alter the chondrogenic activity of donor G115 cultures. This difference could reflect a donor-dependent sensitivity to early dynamic loading or could be due to the differences in the loading protocols used with the two donors for the early loading period. The two donors both showed no change in chondrogenic activity with dynamic loading for the loaded day 7-9 set, although one minor difference was noted at the early harvest time. The stimulation of ^{35}S-sulfate incorporation in the day 10 cultures for donor G117 but not donor G115 could be related to underlying differences in proteoglycan synthesis for the two donors. At day 7, the two donors had comparable ^{35}S-sulfate incorporation rates, but by day 10 donor G115 had a higher ^{35}S-sulfate incorporation rate resulting from an increase in the rate for donor G115 and to no change in the rate for donor G117 (data not shown).

Further experimentation was needed to clarify the apparent discrepancy between donors in response to early dynamic loading. The day 1-3 loading set of the initial loading experiment was subsequently repeated, with evaluation of the chondrogenic activity of day 14 cultures. To more thoroughly explore the potential donor influence on

the response to early loading, a total of four donors were evaluated in parallel, including donors G117 and G115. Dynamic loading did not modulate chondrogenic activity, as ^{35}S-sulfate incorporation, sGAG/DNA, and type II collagen staining were comparable across the loading groups for each donor evaluated (Figure 14, Figure 15). DNA content and ^{3}H-proline incorporation were also comparable across the loading groups for each donor with one exception. For donor G132 the dynamic group exhibited a modest decrease in DNA compared to the 15% static group but not compared to the 0% static group (11%; $p<0.035$). For each donor, the 0% static group was comparable to both the dynamic group and the 15% static group for the parameters measured (0% static data not shown). Comparing the response for donor G117 in this experiment and in the initial experiment, it is concluded that the results for the loaded day 1-3 set in the initial experiment were due to the deviation in the loading protocol. Together, the first two loading experiments demonstrated that oscillatory compressive loading initiated during the first week of culture did not alter the chondrogenic activity of differentiating MPC cultures and the lack of response to dynamic loading was consistent across multiple donors.

Secondary Influences on the MPC Response to Dynamic Loading

A number of factors associated with the loading protocol could influence the ability of the MPCs to exhibit a response to oscillatory compressive loading. In considering the loading protocol, a primary variable was the number of consecutive days that loading was applied. Increasing the number of days that loading was applied could support a more slow-acting, cumulative loading effect on MPC chondrogenic activity that would not be possible with the 3-day loading interval used in the initial study. Furthermore, TGF-β1 in

the culture media during loading could promote MPC chondrogenic activity at such a high level that its presence might mask any weaker stimulatory effects of dynamic loading. An experiment was therefore conducted to assess the influences of longer-term loading and TGF-β1 supplementation on the response to dynamic loading. Loading was initiated on day 4 and applied for 6 consecutive days. Dynamic loading did not change the chondrogenic activity of day 14 MPC cultures (Table 5), in agreement with previous results for the day 4-6 loading interval. The lack of response to dynamic loading was observed for loading in either the presence or absence of TGF-β1. For each media formulation, all loading groups were comparable for ^{35}S-sulfate incorporation and sGAG/DNA, as well as DNA content and ^{3}H-proline incorporation.

In addition to the loading protocol, the culture system could also contribute to how MPCs respond to oscillatory compressive loading. In considering the culture system, a primary variable was the hydrogel utilized in culturing the MPCs. Agarose was a possible alternative hydrogel for these experiments, as it had been used in loading studies with chondroprogenitor cells and isolated chondrocytes [15, 19]. An experiment was conducted to compare the response to early loading (day 1-3) for MPCs suspended in alginate and agarose, with cultures evaluated at day 14. In both alginate and agarose gels, dynamic loading did not modulate the response of the MPC cultures compared to the static groups for either ^{35}S-sulfate incorporation or sGAG/DNA, indicators of chondrogenic activity (Table 6). For agarose cultures, all loading groups were also comparable for DNA content and ^{3}H-proline incorporation. For alginate cultures, the dynamic group had significantly higher ^{3}H-proline incorporation compared to the 15% static group (12%; $p<0.02$) and had significantly lower DNA content compared to the

15% static group (10%; $p<0.03$), but there were no differences between the dynamic group and the 0% static group for these parameters. For each gel type, the two static groups were comparable for all parameters measured.

Comparing Loading Effects on MPC Chondrogenic Activity for Intermediate and Extended Preculture Times

The previous loading studies focused on differentiating MPC cultures and demonstrated a lack of response to oscillatory loading. However, a differentiated MPC culture with substantial matrix accumulation could have a different sensitivity to oscillatory loading effects on chondrogenic activity than the differentiating MPC cultures. A study was conducted to explore this issue and compared loading effects on MPC chondrogenic activity for loading initiated at intermediate and extended preculture times. Loading was applied for 6 consecutive days and gels were harvested the day after loading completion. Concurrent with loading initiation, half of the MPC gels were cultured in media lacking TGF-β1. Based upon an MPC time course study conducted previously, day 7 was selected for the intermediate culture time to represent a differentiating MPC culture with relatively limited matrix accumulation, while Day 21 was selected for the extended preculture period to represent a differentiated MPC culture with increased matrix accumulation. The previous results indicated that sGAG had begun to accumulate but type II collagen staining was limited at day 7, while enhanced sGAG accumulation and extensive type II collagen staining were present at day 21.

Dynamic loading modulated biosynthetic markers to a limited degree in the MPC cultures (Figure 16). For MPC cultures supplemented with TGF-β1, the dynamic Day 7-12 group exhibited an increase in ^{35}S-sulfate incorporation compared to each static group (19% versus 15% static group; 14% versus 0% static group; $p<0.02$) but there were no

differences in sGAG/DNA (data not shown). Also, the dynamic Day 21-26 group exhibited a decrease in ^{3}H-proline incorporation compared only to the 0% static group (14%; $p<0.02$). For MPC cultures where TGF-β1 was removed with loading initiation, the dynamic Day 7-12 group exhibited an increase in ^{35}S-sulfate incorporation compared only to the 15% static group (25%; $p<0.03$) but there were no differences in sGAG/DNA (data not shown). Also, the dynamic group exhibited a decrease in ^{3}H-proline incorporation compared to the static groups for Day 7-12 loading (20% decrease versus 15% static group; 18% decrease versus 0% static group; $p<0.05$) and for Day 21-26 loading (15% decrease versus 15% static group; 15% decrease versus 0% static group; $p<0.005$). sGAG/DNA and DNA were not modulated by dynamic loading at either loading interval for the media conditions evaluated (data not shown). For both media conditions and loading intervals, the two static groups were comparable for the parameters measured.

Discussion

Mechanical preconditioning has been proposed as a stimulatory factor for the *in vitro* development of tissue-engineered cartilage constructs. While adult MPCs are a promising cell source for cartilage tissue engineering applications, little is known about the ability of mechanical signals to influence MPC chondrogenic differentiation and matrix accumulation. Assessing whether mechanical signals can be used to promote matrix production in differentiating MPCs in an *in vitro* culture system has important implications for cartilage tissue engineering strategies. This work evaluated oscillatory compressive loading effects on the chondrogenic activity of MPCs undergoing chondrogenic differentiation in alginate suspension, with chondrogenic activity

representing an assessment of cartilage matrix production focusing on proteoglycan synthesis and sGAG accumulation.

Oscillatory unconfined compressive loading of MPC/alginate gels initiated during the first seven days of culture and combined with TGF-β1 supplementation did not enhance chondrogenic activity above the level supported by TGF-β1 alone. The length of the preculture period preceding loading initiation did not affect the response to dynamic loading, as three distinct culture times were evaluated and dynamic loading did not alter subsequent chondrogenic activity in any of the cultures. To assess whether loading effects would enhance chondrogenic activity in a manner that was beneficial to the development of MPC-based tissue engineered implants, the evaluation of chondrogenic activity focused on the production of cartilage typical matrix components rather than gene expression changes and the experimental design emphasized sustained loading effects rather than very early loading effects. Identification of an MPC culture showing enhanced chondrogenic activity with loading would have been followed by additional studies evaluating gene expression of appropriate matrix proteins. However, evaluation of changes in gene expression in response to loading in the absence of corresponding changes in matrix accumulation would have had limited usefulness and was therefore not pursued given the results of the initial loading experiments.

Two recent studies focusing on chondrogenic differentiation have demonstrated stimulatory effects for dynamic loading of MPC cultures. The application of oscillatory compressive loading (4 hours/day) during the first week of culture notably increased aggrecan, type II collagen, and type I collagen gene expression and increased sGAG and hydroxyproline content over unloaded controls in day 14 cultures of human MPCs seeded

in a hyaluronanester-gelatin composite and cultured with chondrogenic supplements [190]. Differences in experimental variables between this study and the work presented in this chapter, including the scaffold/gel material and the number of loading days, might explain in part the differences in results with regard to loading effects on sGAG accumulation. Daily oscillatory compressive loading (4 hours/day) stimulated aggrecan and type II collagen gene expression over no-load controls in rabbit MPC/agarose constructs cultured in the absence of chondrogenic supplements [188]. Importantly, changes in gene expression might not correlate to similar changes in protein expression, so direct comparison of the rabbit MPC study to our goat MPC experiments is not possible.

The experimental approach taken in this work focused on evaluating MPC chondrogenic activity in a culture system, as related to the scaffold material and culture media, which was relevant to potential cartilage tissue engineering strategies. The use of TGF-β1 supplementation to support chondrogenic differentiation and to maintain chondrogenic cultures in a serum-free media was therefore central to this approach. Since previous experimentation had established the stimulatory effects of various growth factors, including TGF-β1, for MPC chondrogenic differentiation and sustained long-term culture, *in vitro* cartilage tissue engineering strategies utilizing MPCs would likely depend first and foremost upon the simpler strategy of chemical stimulation rather than the more complicated strategy of mechanical stimulation. Thus, for the loading results to be consequential with respect to possible *in vitro* tissue engineering strategies, the benefit of mechanical stimulation would need to be proven in the presence of TGF-β1 or some other appropriate chemical stimuli. Evaluating loading effects in the presence of growth

factors would also be more representative of the complex environment present during *in vivo* tissue development or regeneration.

There are conflicting reports in the literature on combining mechanical stimulation and growth factor supplementation to support MPC differentiation and matrix accumulation. Human MPCs cultured in the presence of chondrogenic supplements responded positively to dynamic compression and dynamic hydrostatic pressure [187, 190, 192], while rabbit MPCs cultured in the absence of TGF-β1 showed a much greater stimulatory response to dynamic compression than cells cultured in the presence of TGF-β1 [188]. The differences between these studies may suggest possibly important species differences regarding the interaction of TGF-β1 supplementation and the MPC response to mechanical stimulation. Overall, conducting loading experiments on MPC cultures that had received no exposure to TGF-β1 would have been irrelevant to the underlying motivations of this work. However, the lack of effect of dynamic compression observed in this work could be due in part to the dose of TGF-β1 used in these experiments. The chondrogenic activity of MPCs exposed to continuous 10ng/ml TGF-β1 supplementation could have been at a near-saturation level and unable to be further positively modulated by the applied mechanical signals. However, our observation that cultures either exposed to or lacking TGF-β1 during loading exhibited a lack of modulation in chondrogenic activity in response to oscillatory loading initiated on day 4 may indicate that other variables in the experimental design had a greater impact on the loading results than did media supplementation.

Another possible explanation for the lack of effect of oscillatory compressive loading observed in this work is that the combination of loading mode, loading parameters, and

gel material utilized in these experiments provided insufficient mechanical stimulation of the MPCs. Properties of the alginate gel, including mechanical and surface characteristics, will determine how the macroscopic unixial compressive deformation applied to the cylindrical gels is transferred to the cellular microenvironment. Importantly, suspended cells cannot specifically interact with the surrounding alginate [193]. Potential sources of mechanical stimulation resulting from compressive loading of early MPC/alginate cultures with limited matrix accumulation include matrix-independent cell deformation and fluid pressurization. Gross static compression of BAC/alginate gels resulted in cellular deformation and a change in the nuclear morphology [194]. A recent preliminary study with human MPCs in alginate showed enhanced aggrecan gene expression and type II collagen deposition with exposure to TGF-β1 and cyclic hydrostatic pressurization [192]. However, the levels of cellular deformation and fluid pressurization associated with dynamic compression at the peak amplitude of 3% utilized in these experiments may have been inadequate to activate signal transduction pathways within the isolated cells.

The demonstration that MPCs in alginate and agarose both showed a lack of response to oscillatory compressive loading initiated on culture day 1 suggests that the response of very early MPC cultures to compressive loading is not restricted to culture in alginate suspension but is more generally indicative of culture in hydrogels that do not support specific interactions with the suspended cells. Specific interactions between MPCs and the 3-D gel or scaffold in which the cells reside may be a potentially important mediator of mechanotransduction in cultures where extracellular matrix accumulation is limited. In contrast to our work with alginate gels, oscillatory compressive loading of MPC-

seeded hyaluronanester-gelatin composite scaffolds was reported to support sustained enhancement of matrix synthesis [190]. One approach for exploring possible limitations of the current alginate culture system would be to utilize a hydrogel that incorporates adhesion sequences, such as RGD-functionalized alginate or agarose with covalently immobilized adhesion peptides [195, 196].

Secondary to the studies focusing on differentiating MPC cultures, one study compared loading effects on MPC chondrogenic activity for loading initiated at intermediate (day 7) and extended (day 21) preculture times. Oscillatory compressive loading influenced biosynthesis rates to a limited degree but did not support changes in matrix accumulation of the MPC cultures. Oscillatory compression applied during the second week of culture increased proteoglycan synthesis, but this effect was absent for loading of MPCs during the fourth week of culture. Pericellular matrix accumulation with increasing time in culture could have limited the ability of MPCs to respond to mechanical loading at the later culture time, as the pericellular matrix may shield the cells from deformation in alginate gels similar to observations in agarose cultures [197]. The modulation in proteoglycan synthesis did not correlate with enhanced sGAG/DNA accumulation in the day 13 cultures, which could indicate that the increase in ^{35}S-sulfate incorporation observed after loading completion was not indicative of ^{35}S-sulfate incorporation levels present throughout the loading period. Alternatively, the level of increase noted in ^{35}S-sulfate incorporation may have been insufficient to appreciably alter the level of sGAG/DNA accumulation. Identifying an oscillatory compressive loading protocol that enhanced matrix deposition in primary articular chondrocyte cultures was found to be challenging in previous studies. A long-term loading protocol notably

influenced the mechanical properties of the accumulating matrix in articular chondrocyte/agarose cultures, but enhancement of sGAG and hydroxyproline content was noted only after three weeks of loading [15]. A recent investigation of short-term loading effects found that utilization of an alternate-day loading period stimulated proteoglycan synthesis but inhibited protein synthesis in articular chondrocyte/agarose cultures in low-serum media [99].

The loading experiments in this chapter do not address the possibility that long-term development of tissue-engineered cartilage constructs utilizing MPCs could benefit from a sustained oscillatory compressive loading protocol applied on the order of several weeks. Sustained loading could support improved matrix organization or could promote small changes in matrix production that, when summed over many days, could lead to enhanced matrix accumulation. Our results indicate that no benefit with regard to matrix production would be gained by loading MPC/alginate gels during the first week of culture, but it is possible that loading during the earliest phase of matrix accumulation could have unexpected benefits if included in a multi-week loading protocol for long-term tissue development. Furthermore, differentiating MPC cultures may be able to respond to mechanical stimulation in discrete, transient ways that would not have been detected with the experimental protocol used in these loading experiments, since the response to loading was evaluated following an extended period after loading completion.

In conclusion, oscillatory unconfined compressive loading of MPC/alginate cultures in chondrogenic media did not enhance the chondrogenic activity of differentiating MPCs above the level supported by chondrogenic media alone for loading applied 3 hours/day for three to six consecutive days. The length of the preculture period preceding loading

initiation, ranging from one to seven days, did not alter the lack of effect for oscillatory loading. Furthermore, extending the preculture period to three weeks and thereby supporting enhanced matrix accumulation prior to loading also did not support a modulation of chondrogenic activity with oscillatory compressive loading. While further research is needed to fully assess the overall utility of a mechanical preconditioning strategy based upon oscillatory compressive loading of MPCs, our results suggest that the enhancement of matrix production in MPC/hydrogel cultures requires that loading be conducted for greater than one week when loading is initiated during the first, second or fourth weeks of culture.

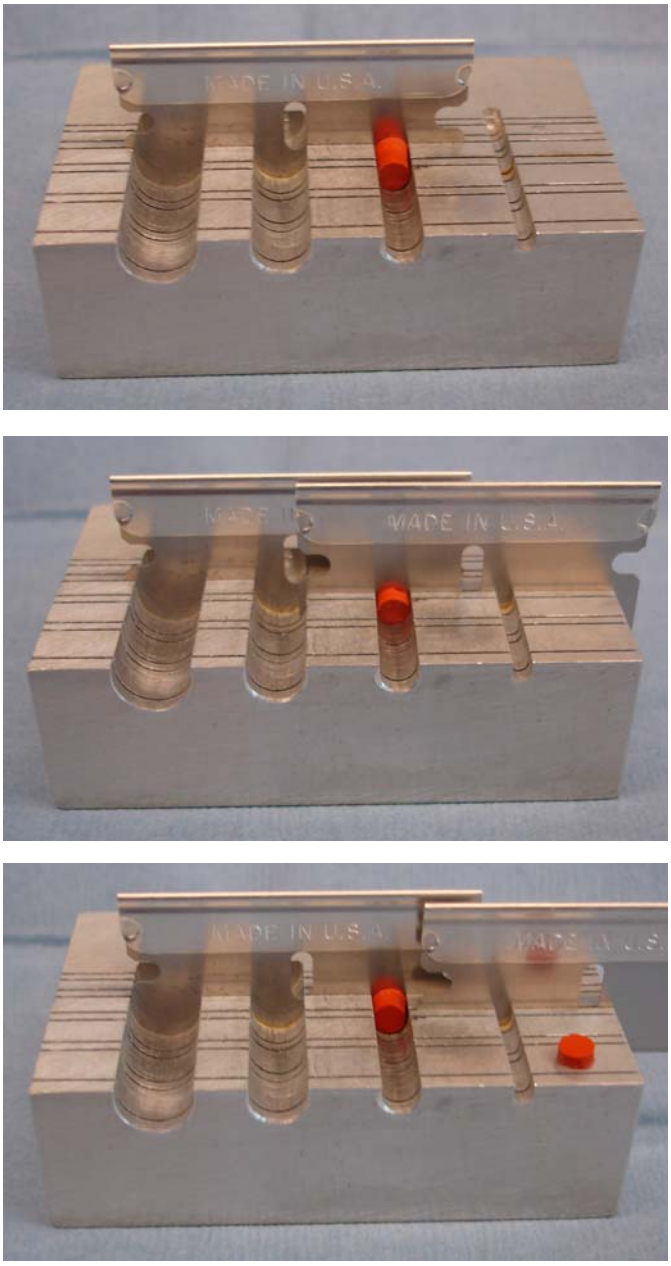


Figure 8: Gel Sizing Tool. Top: A gel is placed on its side into a 4mm diameter groove, which has slits running perpendicular to the groove, and the gel is positioned against a razor blade that sits in the designated slit. Middle: A second razor blade is placed into the adjacent designated slit, which is situated 3mm away from the first razor blade. Bottom: The blade cuts off the excess portion of the gel, resulting in a gel of height 3mm.

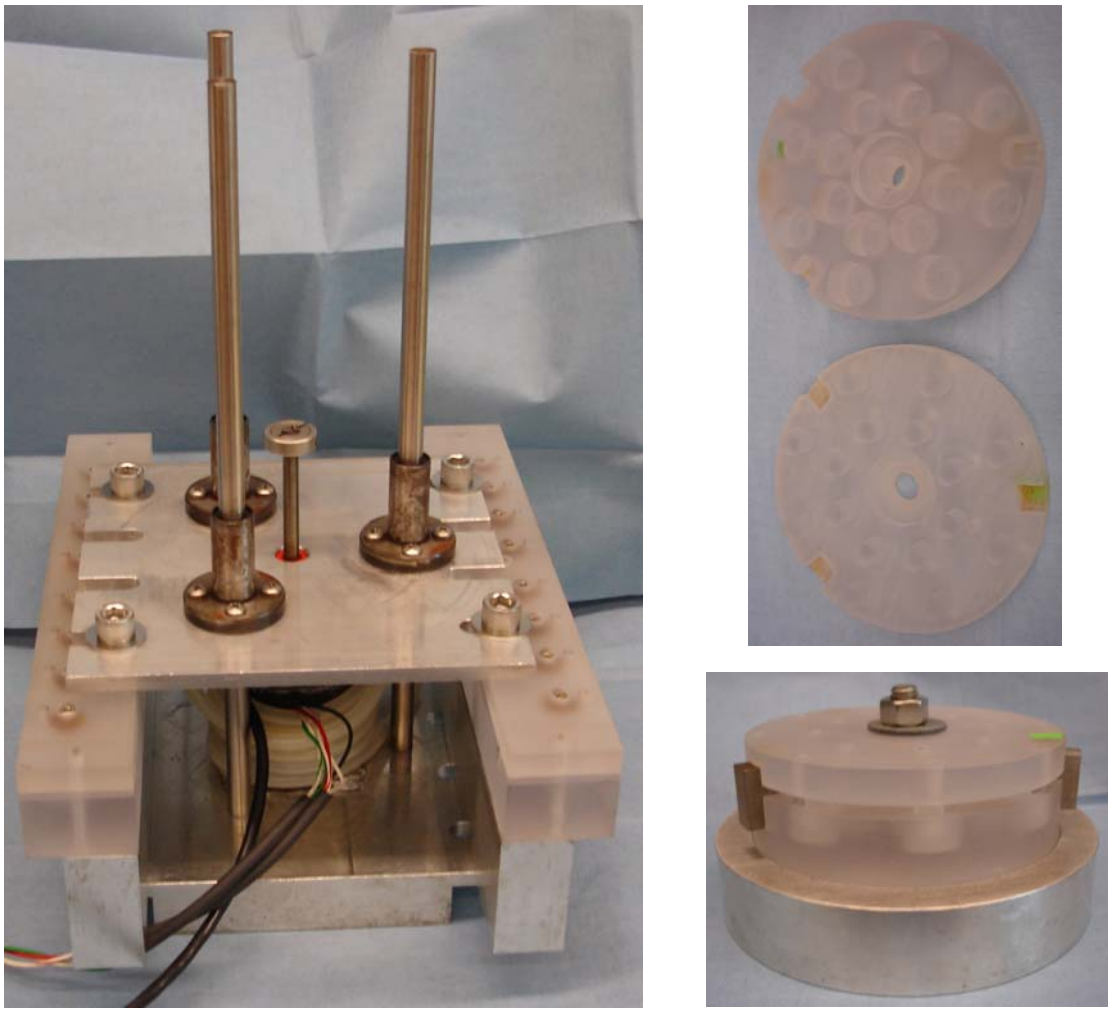


Figure 9: Loading System & Reactors for Chondrogenic Activity Experiments. Left: Compression device (i.e. dynamic loading system) used to apply controlled deformation to gels situated within the bioreactor cassette via stainless steel platens located on the bottom surface of the bioreactor lid. The bioreactor lid is attached to the top plate of the loading frame. Vertical movement of this top plate results from lead screw rotation of a linear stepper motor. Right: Disassembled (top) and assembled (bottom) static compression bioreactor.

Table 4: Summary of Studies Evaluating Oscillatory Compressive Loading Effects on MPC Chondrogenic Activity. For study 1, cultures were evaluated either the day after loading completion or on day 14. For study 2, cultures were evaluated on day 14. For study 3, media supplementation (± TGF-β1) was a secondary variable of interest and all cultures were evaluated the day after loading completion.

	Experimental Variable	Levels Evaluated	Number of Loading Days
Study 1	Day of Loading Initiation	Day 1, Day 4, or Day 7	3
Study 2	1) Media Supplementation 2) Hydrogel Material	± TGF-β1 Alginate or Agarose	6 3
Study 3	Day of Loading Initiation	Day 7 or Day 21	6

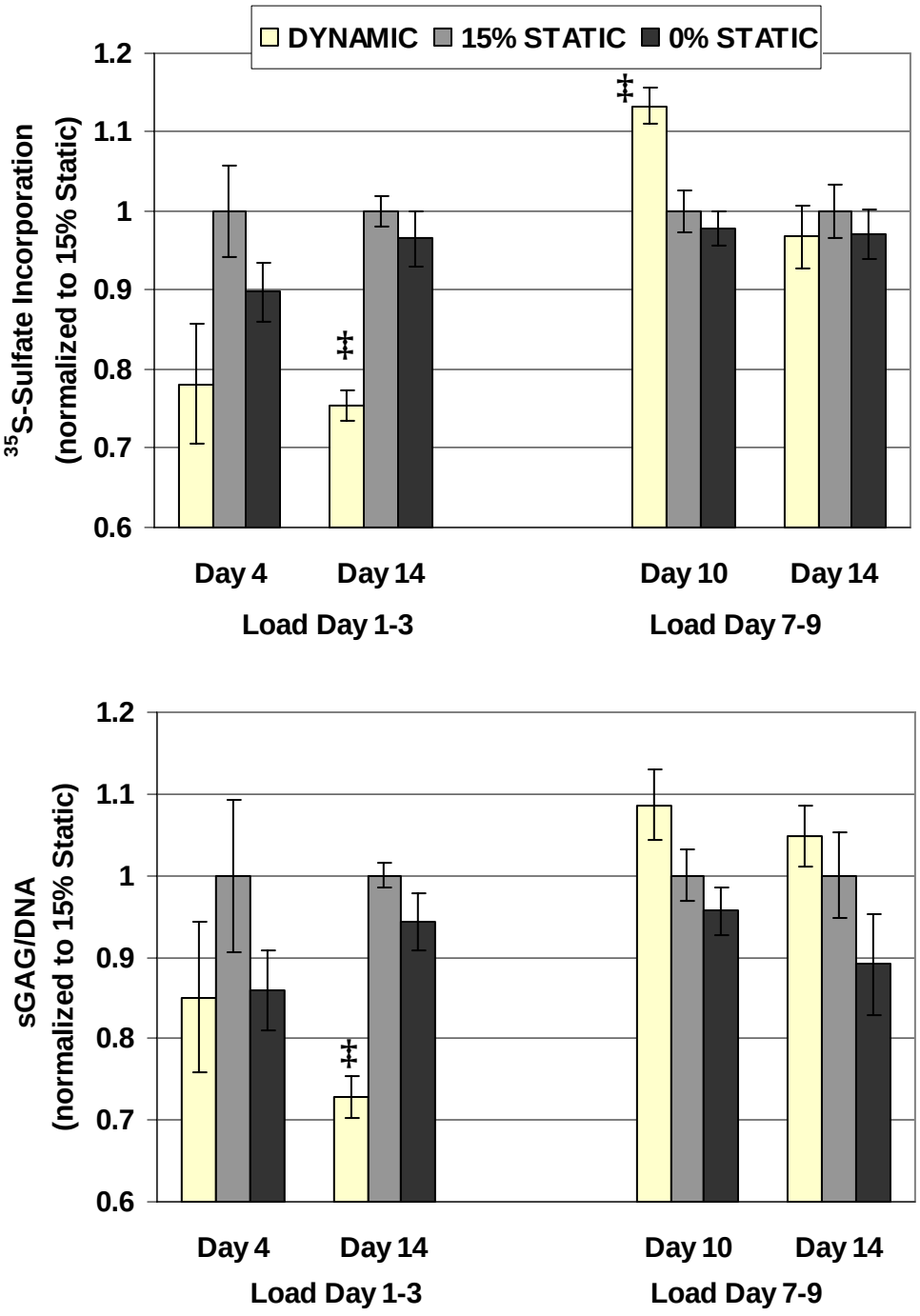


Figure 10: Evaluation of Chondrogenic Activity in Donor G117 Cultures Subjected to Three-Day Compressive Loading. Dynamically loaded gels (15 ± 3% compression; 1 Hz) were compared to gels held at 15% static compression and gels held at 0% static compression with loading for 3 hours each day. Cultures were evaluated on the day following loading completion or on day 14. Values are mean ± SEM (n=6gels/loading group; 1 experiment). ‡ denotes significantly different versus static groups for the designated culture time.

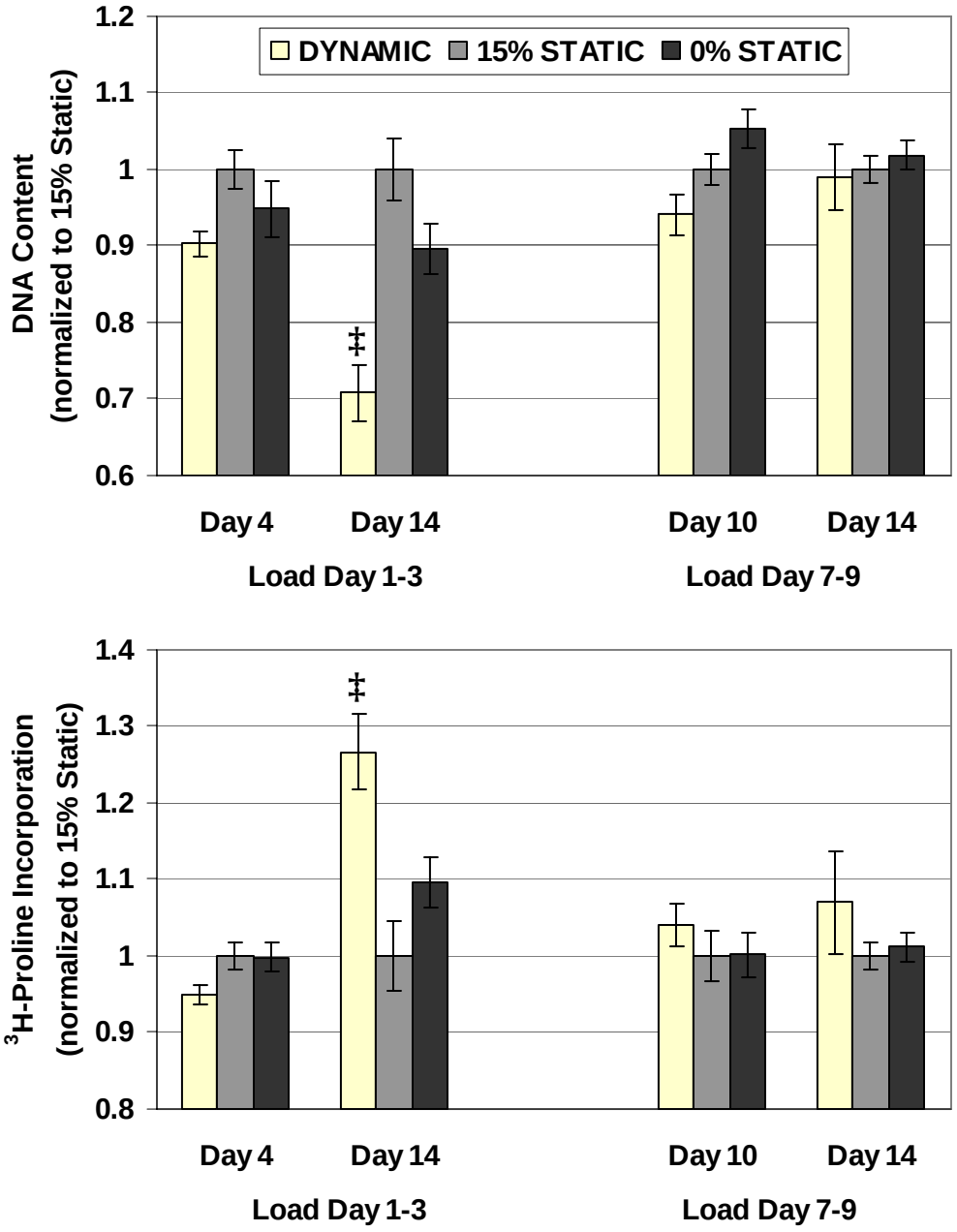


Figure 11: Evaluation of Donor G117 Cultures Subjected to Three-Day Compressive Loading. Dynamically loaded gels (15 ± 3% compression; 1 Hz) were compared to gels held at 15% static compression and gels held at 0% static compression with loading for 3 hours each day. Cultures were evaluated on the day following loading completion or on day 14. Values are mean ± SEM (n=6gels/loading group; 1 experiment). ‡ denotes significantly different versus static groups for the designated culture time.

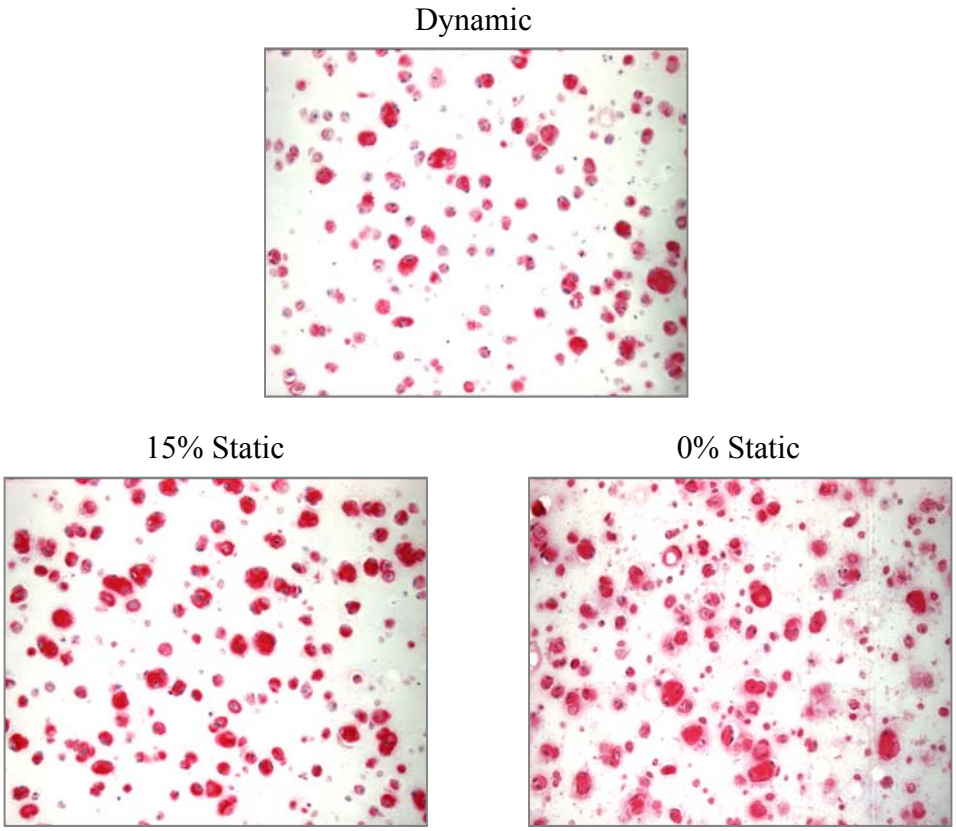


Figure 12: Type II Collagen Staining of Day 14 G117 Cultures Loaded on Days 1-3. Type II collagen immunostaining (20X; red coloration indicates presence of antigen) of gels subjected to dynamic compression (15±3% compression at 1 Hz), 15% static compression, or 0% static compression for 3 hours/day for 3 consecutive days beginning on culture day 1 and then cultured to day 14.

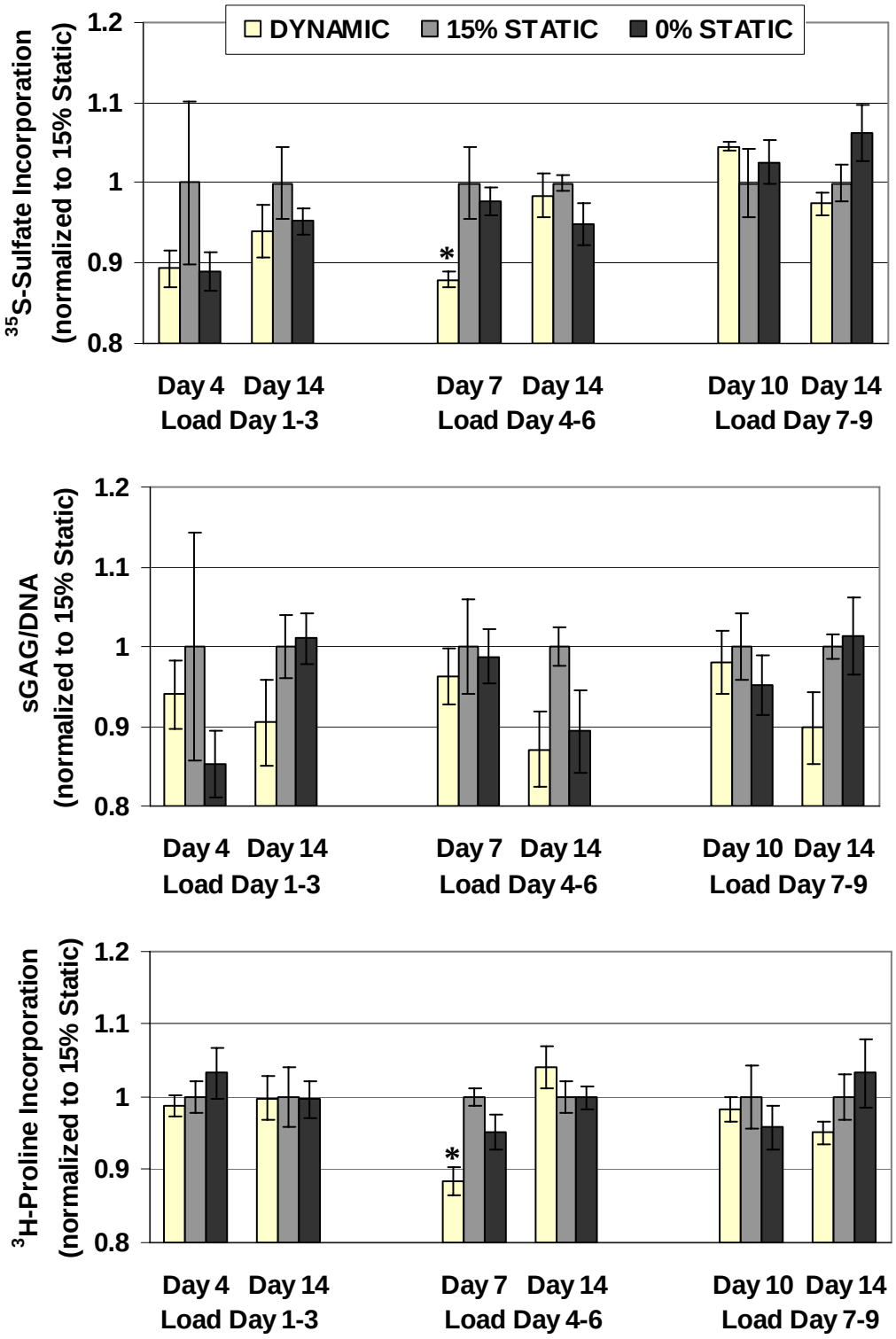


Figure 13: Evaluation of Donor G115 Cultures Subjected to Three-Day Compressive Loading. Cultures (dynamically loaded at 1 Hz or statically loaded to 15% or 0% for 3hr/day) were evaluated on the day following loading or on day 14. (Mean ± SEM; n=6gels/loading group; 1 experiment). ***** denotes significantly different vs. 15% static for the designated culture time.

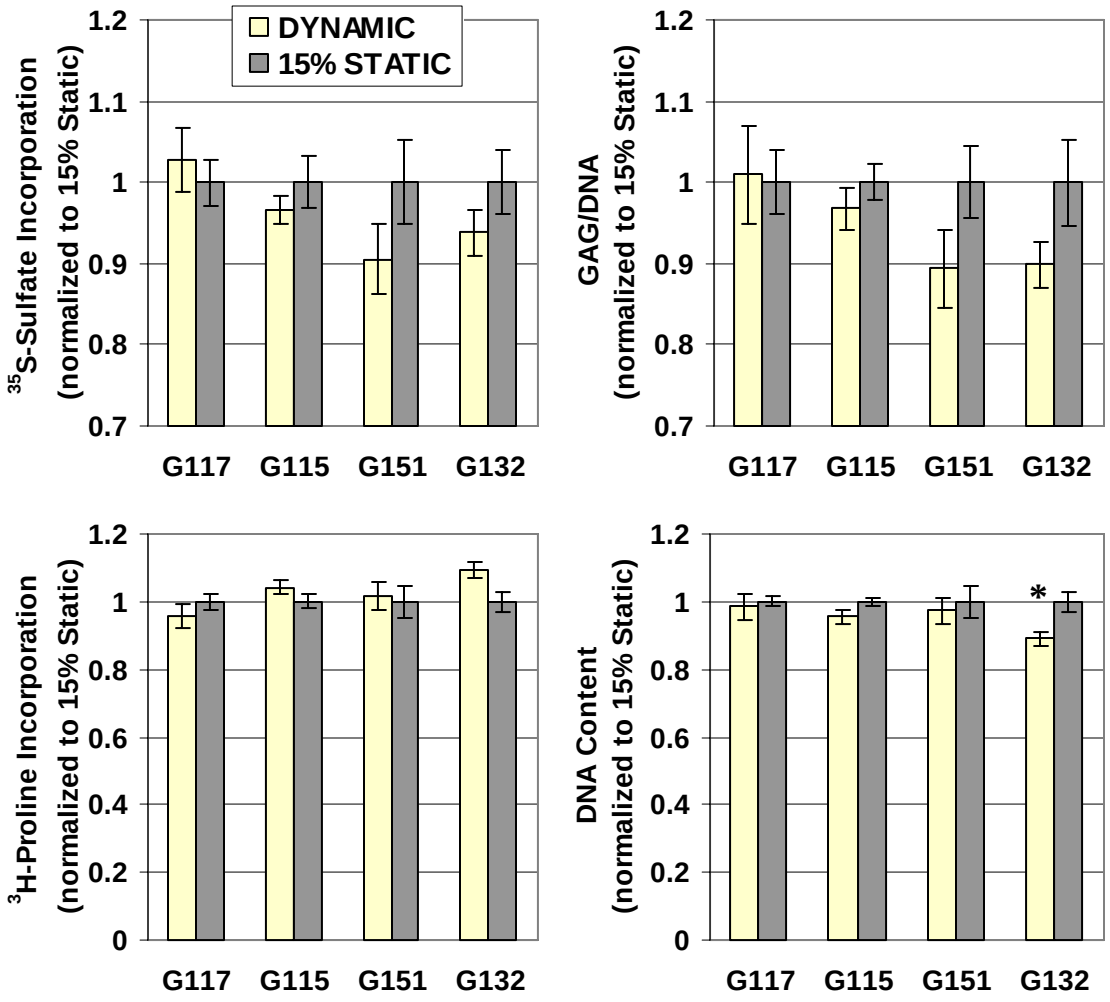


Figure 14: Comparison of the MPC Donor Response to Compressive Loading for Cultures Loaded on Days 1-3. Dynamically loaded gels (15 ± 3% compression; 1 Hz) were compared to gels held at 15% static compression and gels held at 0% static compression with loading for 3 hours each day. Cultures were evaluated on day 14 for four MPC donors (donor G117, etc). Values are mean ± SEM (n=6gels/loading group; 1 experiment). ***** denotes significantly different vs. 15% static for the designated donor. For each donor, the 0% static group was comparable to both the dynamic group and the 15% static group for all parameters measured (0% static data not shown).

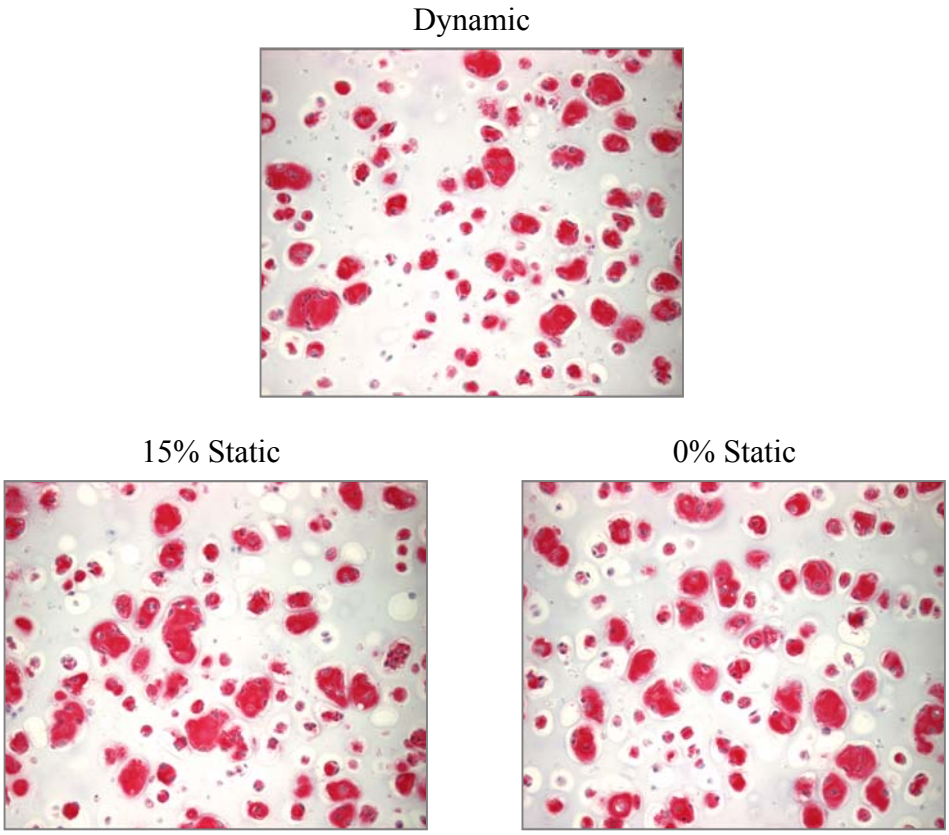


Figure 15: Type II Collagen Staining of Day 14 MPC Cultures Loaded on Days 1-3. Type II collagen immunostaining (20X; red coloration indicates presence of antigen; images shown for donor G117) of gels subjected to dynamic compression (15±3% compression at 1 Hz), 15% static compression, or 0% static compression for 3 hours/day for 3 consecutive days beginning on culture day 1 and then cultured to day 14.

Table 5: Comparison of TGF-β1 Supplementation on the Response to Loading on Days 4-9. Gels were subjected to dynamic compression (15±3% compression at 1 Hz), 15% static compression, or 0% static compression for 3 hours/day. Cultures were evaluated on day 14. TGF-β1 was either included in the culture media for the duration of the experiment (MPC+TGF-β1) or was excluded concurrent with loading initiation (MPC). Values are mean ± SEM (n=6gels/loading group; Donor G117; 1 experiment).

Parameter (normalized to 15% Static)	^{35}S-Sulfate Incorporation	sGAG/DNA	DNA	^{3}H-Proline Incorporation
MPC + TGF-β1				
Dynamic	1.006 ± 0.013	1.007 ± 0.023	0.975 ± 0.027	1.049 ± 0.026
15% Static	1.000 ± 0.025	1.000 ± 0.028	1.000 ± 0.046	1.000 ± 0.054
0% Static	1.049 ± 0.041	1.067 ± 0.045	1.014 ± 0.043	0.992 ± 0.032
MPC				
Dynamic	0.992 ± 0.148	0.924 ± 0.084	0.989 ± 0.036	0.959 ± 0.118
15% Static	1.000 ± 0.050	1.000 ± 0.039	1.000 ± 0.040	1.000 ± 0.035
0% Static	1.060 ± 0.039	1.041 ± 0.036	0.981 ± 0.028	1.017 ± 0.029

Table 6: Comparison of Hydrogel Materials on the Response to Loading on Days 1-3. MPCs were suspended in alginate or agarose, then gels were subjected to dynamic compression (15±3% compression at 1 Hz), 15% static compression, or 0% static compression for 3 hours/day. Cultures were evaluated on day 14. Values are mean ± SEM (n=6gels/loading group except n=4 for agarose dynamic; Donor G117; 1 experiment). ***** denotes significantly different vs. 15% static for the designated gel material.

Parameter (normalized to 15% Static)	^{35}S-Sulfate Incorporation	sGAG/DNA	DNA	^{3}H-Proline Incorporation
Alginate				
Dynamic	1.050 ± 0.019	1.060 ± 0.020	* 0.899 ± 0.024	* 1.117 ± 0.027
15% Static	1.000 ± 0.022	1.000 ± 0.021	1.000 ± 0.023	1.000 ± 0.015
0% Static	1.025 ± 0.023	1.058 ± 0.035	0.954 ± 0.026	1.030 ± 0.035
Agarose				
Dynamic	0.739 ± 0.063	0.726 ± 0.113	0.971 ± 0.019	0.892 ± 0.047
15% Static	1.000 ± 0.077	1.000 ± 0.091	1.000 ± 0.072	1.000 ± 0.030
0% Static	0.917 ± 0.055	0.906 ± 0.102	0.904 ± 0.055	0.994 ± 0.036

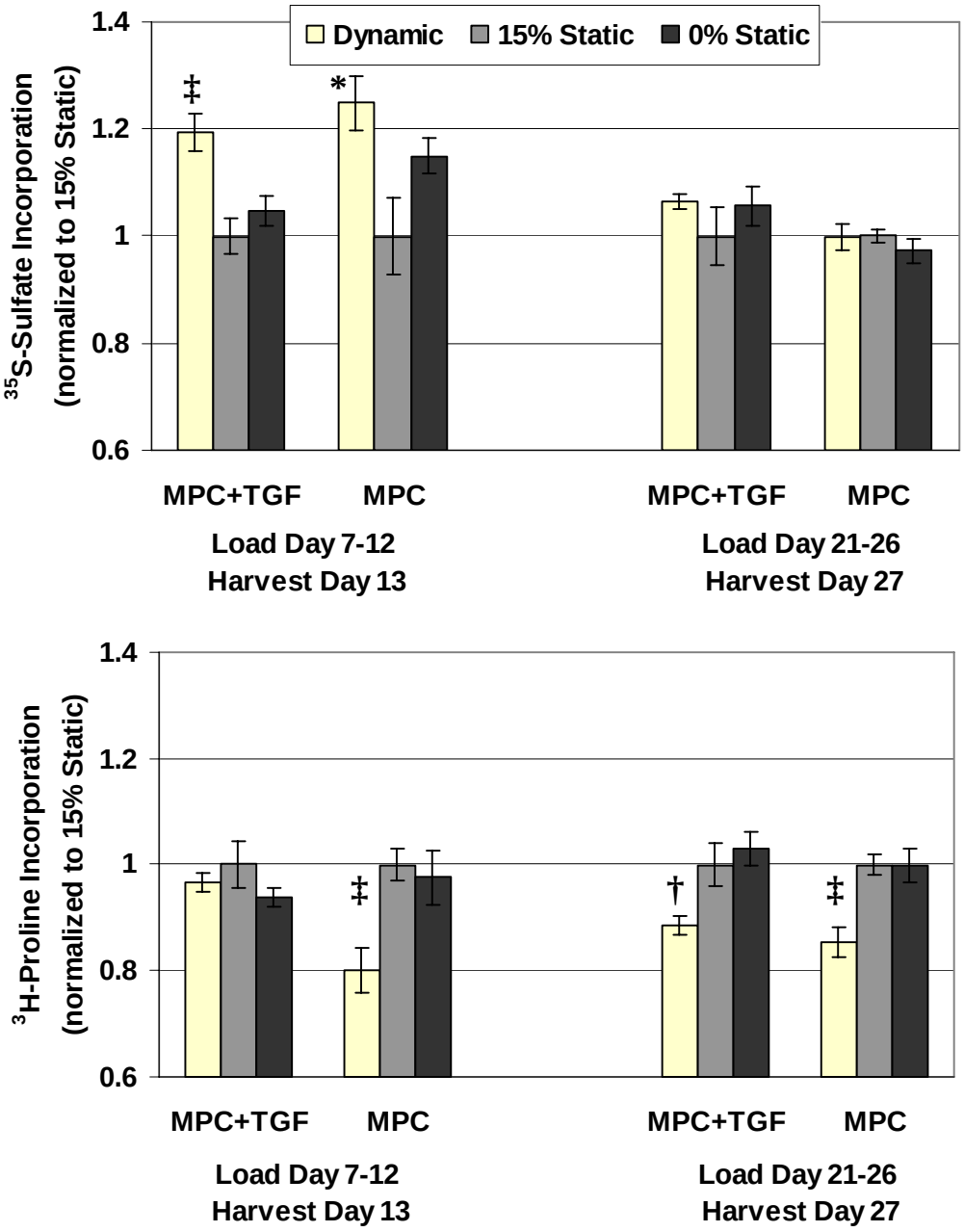


Figure 16: Comparison of Compressive Loading Effects for MPC Cultures at Intermediate and Extended Preculture Times. Gels were subjected to dynamic compression (15±3% compression at 1 Hz), 15% static compression, or 0% static compression for 3 hours per day for 6 consecutive days beginning on culture day 7 or day 21. TGF-β1 was either included in the MPC culture media for the duration of the experiment (MPC+TGF) or was excluded concurrent with loading initiation (MPC). Values are mean ± SEM (n=6gels/loading group/cell type except n=4 for day 13 dynamic; 1 experiment). ‡ denotes significantly different vs. static groups for the designated cell type. ***** denotes significantly different vs. 15% static for the designated cell type. † denotes significantly different vs. 0% static for the designated cell type.

CHAPTER 5

EVALUATION OF MPC BIOSYNTHESIS DURING CONTINUOUS OSCILLATORY COMPRESSIVE LOADING

Introduction

Orthopaedic tissues, such as bone and cartilage, are subjected to frequent and repeated bouts of mechanical loading with normal activity. Evaluations at the tissue level have shown that mechanical stimulation or the lack thereof can alter tissue composition. In bone, experimental models of disuse result in a loss of bone mineral density and exercise above normal activity levels may be beneficial for recovery of bone mineral density with remobilization [198, 199]. Likewise, experimental studies have indicated that exercise leads to increased proteoglycan content in articular cartilage, while joint immobilization leads to a loss of proteoglycan content that can be recovered with restoration of joint mobility [58].

These changes in tissue composition are ultimately the result of changes in metabolism of individual cells. To explore linkages between mechanical stimulation and changes in cellular activities, *in vitro* studies have been conducted on isolated cells cultured in a variety of systems. Proteoglycan synthesis, type II collagen gene expression, and aggrecan gene expression have been shown to be modulated in isolated chondrocytes by various loading protocols evaluating compression and hydrostatic pressure [24, 61, 63, 66, 67]. Osteoblast proliferation, alkaline phosphatase activity, and osteopontin gene expression have been demonstrated to change in response to fluid flow and strain [200-204].

While much attention has focused on evaluating the response to mechanical stimulation in differentiated cells isolated from mature tissue, multipotential progenitor cells and lineage-committed progenitor cells will also be subjected to mechanical stimulation during orthopaedic tissue development and repair. There is limited evidence to indicate that forms of mechanical loading shown to modulate differentiated cells may also influence responses in progenitor populations. Rat bone marrow stromal cells showed enhancement of alkaline phosphatase expression with application of cyclic strain [205]. Human marrow stromal cells subjected to oscillatory fluid flow exhibited increased proliferation and increased osteopontin and osteocalcin mRNA levels [145]. Increased gene expression for the chondrogenic markers aggrecan and type II collagen was observed with oscillatory compressive loading of rabbit mesenchymal stem cells [188]. A similar response was noted for hydrostatic pressurization of bovine bone marrow stromal cells [146]. Together these studies provide direct evidence that progenitor cells exhibit mechanoresponsiveness.

Mesenchymal progenitor cells (MPCs) have been identified as a potential cell source for orthopaedic tissue engineering strategies. Given the mechanically complex environment into which these cells may be placed, understanding how MPCs, both as undifferentiated and differentiating populations, respond to mechanical stimulation is important. Determining how MPCs respond to types of loading known to modulate the response of differentiated cells is one area of necessary investigation. The primary focus of this chapter was to evaluate MPC biosynthesis during continuous oscillatory compressive loading of MPC/alginate cultures. The mechanoresponsiveness of MPCs undergoing chondrogenic differentiation was evaluated for a loading protocol shown

previously to enhance biosynthesis of chondrocytes in hydrogel suspension [24]. Our *hypothesis* was that oscillatory compressive mechanical loading would stimulate proteoglycan and protein synthesis in differentiating MPC cultures, but would not modulate biosynthesis in prechondrogenic MPC cultures. Oscillatory unconfined compressive loading was applied continuously for a single 20 hour period to gels that were being cultured in media containing radiolabeled sulfate (for proteoglycan synthesis) and proline (for protein synthesis) compounds. For all groups, the effects of loading were determined by comparing radiolabel incorporation rates in dynamically loaded constructs to static control constructs. MPCs were cultured in media containing TGF-β1 to support chondrogenic differentiation. Loading was initiated at culture times (days 1, 4, and 7) demonstrated in Aims 1 and 2 to correspond to distinct MPC cultures, as distinguished by increasing levels of proteoglycan synthesis and sGAG accumulation with increasing time in culture.

No previous reports were available for radiolabel-oscillatory compression studies utilizing alginate suspension of isolated chondrocytes at early cultures times. Therefore, bovine articular chondrocytes (BACs) were also subjected to the described loading protocol to provide a baseline assessment of primary articular chondrocyte mechanoresponsiveness in alginate suspension. A primary goal of the initial loading study was to identify a loading protocol that would stimulate proteoglycan synthesis in day 1 BAC/alginate cultures, similar to previous results in BAC/agarose cultures. The loading frequency level was found to be a critical parameter influencing mechanoresponsiveness of both MPC cultures and day 1 BAC cultures

Methods

Materials

Goat MPCs were provided by Osiris Therapeutics, Baltimore, MD. Bovine stifle joints were from Research 87, Marlborough. MA. All media, trypsin, PBS, culture additives (unless noted otherwise), and pepsin were from Invitrogen Life Technologies, Chicago, IL. FBS was from JRH Biosciences, CA, for the MPC cultures and was from Hyclone, Logan, UT, for the BAC cultures. Ascorbic acid-2-phosphate, L-proline, papain, DMMB, chondroitin sulfate A, and buffer reagents for the biochemical analyses were from Sigma-Aldrich, St. Louis, MO. ITS^{+} premix was from BD Biosciences, Bedford, MA. Recombinant human TGF-β1 and recombinant human FGF basic were from R&D Systems, Minneapolis, MN. Pronova UP LVG alginate was from NovaMatrix, FMC BioPolymer, Norway. Type II collagenase was from Worthington Biochemicals, Lakewood, NJ. L-5-^{3}H-proline was from Amersham Biosciences, Piscataway, NJ. ^{35}S-sodium sulfate and Ecolume were from MP Biomedical, Irvine, CA. The PicoGreen kit was from Molecular Probes, Eugene, OR.

MPC Isolation and Expansion

Osiris Therapeutics conducted the goat MPC isolation from iliac crest bone marrow aspirates followed by adherent primary expansion as described in their published methods for human MPCs [38]. At a later time, passage 0 MPCs were thawed, expanded for one passage, and then the passage 1 MPCs were cryopreserved. To initiate each loading experiment, passage 1 MPCs were thawed and expanded for one additional passage before being cast into alginate gels. Expansion from passage 0 to passage 2 (2700cells/cm^2 seeding density) was conducted in low glucose DMEM with 110mg/L

sodium pyruvate and 584ml/L glutamine containing 10% FBS, 1% antibiotic-antimycotic (100units/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericinB), and 1ng/ml FGF-2. When cells had reached a near-confluent density, the flasks were incubated with 0.42g/L trypsin, 0.44mM EDTA·4Na in Ca^{2+}-free, Mg^{2+}-free PBS followed by rinsing in standard expansion media to release the cells.

3-D MPC Culture

Passage 2 MPCs were suspended in one part Ca^{2+}-free, Mg^{2+}-free PBS and mixed with 2 parts 3% Pronova UP LVG alginate (prepared in Ca^{2+}-free, Mg^{2+}-free PBS and filter sterilized) to yield a cell suspension of $12.5x10^6$cells/ml. 45μl of the cell/alginate suspension was transferred to individual 4mm diameter wells of a custom-designed casting mold. The casting mold consisted of four pieces (from bottom to top): solid polycarbonate base, wire mesh, grade #2 Whatman filter paper, and top polycarbonate piece with a 4x4 grid of 4mm diameter holes drilled out. The filter paper was prewet with the polymerization solution (102mM $CaCl_2$, 10mM Hepes, 100mM NaCl) prior to addition of the cell suspension. After ~25 minutes of incubation, polymerization solution was added to the top of the wells. Cylindrical gels were removed from the wells after a total incubation of ~60 minutes and incubated in 150mM NaCl for ~40 minutes, followed by incubation in chondrogenic media (see below) excluding the ascorbic acid-2-phosphate and TGF-β1 for ~40 minutes. Gels were then transferred to 48-well plates and, unless noted otherwise, cultured throughout the study in chondrogenic media: high glucose DMEM with 110mg/L sodium pyruvate and 584ml/L glutamine containing 1% ITS^+ premix (6.25μg/ml insulin, 6.25μg/ml transferrin, 6.25ng/ml selenious acid, 1.25mg/ml bovine serum albumin, 5.35mg/ml linoleic acid), 1% antibiotic-antimycotic,

40μg/ml L-proline, 50μg/ml ascorbic acid-2-phosphate, and 10ng/ml TGF-β1. Cultures received fresh media changes every 1-2 days.

BAC Isolation and 3-D Culture

BACs were cultured in parallel with the goat MPCs for comparison. The BACs were isolated from femoral cartilage (patellar groove and condyles) of immature calves (~2-4 weeks old) by type II collagenase digestion (520units/ml in high glucose DMEM with 5ml/L neomycin, 10ml/L kanamycin, and 10ml/L antibiotic-antimycotic) for ~42 hours. The isolated chondrocytes were combined with 90% FBS and 10% DMSO, transferred to cryovials, placed in a controlled freezing container, and after ~2 days at -80ºC transferred to LiN_2 storage. Thawed BACs were seeded in 2% alginate at $12.5x10^6$cells/ml as described above for MPCs. The BAC-alginate constructs were cultured in high glucose DMEM containing 10% FBS, 1% antibiotic-antimycotic, 40μg/ml L-proline, and 50μg/ml ascorbic acid-2-phosphate. Cultures received fresh media changes every 1-2 days. BAC cultures were grown in serum-containing media to match previous radiolabel-oscillatory compression studies with BACs in hydrogel suspension [24, 62].

Compressive Loading and Radiolabel

For loading initiation following the designated preculture period, gels were sized to 3mm thickness using a custom-designed sizing tool (Figure 8), transferred to bioreactors, fed with standard culture media (described previously for each cell type) additionally supplemented with 20 μCi/ml L-5-^{3}H-proline and 10 μCi/ml ^{35}S-sodium sulfate, and subjected to 20 hours of unconfined compressive deformation (15±3% compression at a frequency ranging from 0.1-1.0 Hz as specified for each experiment). Control gels were transferred to comparable bioreactors, fed with media containing radiolabel, and

subjected to 20 hours continuous unconfined static deformation (15% compression). At the end of the 20 hour loading period, gels were transferred to 48-well plates containing 1 ml of radiolabel quench solution (PBS additionally containing 0.8mM Na_2SO_4 and 1.0mM L-proline) per well. Samples were washed in a total of four fresh aliquots of quench solution for ~30 minutes per wash. Samples were then transferred to preweighed tubes, weighed, and stored at -20ºC until further processing.

There were no previous reports of compressive loading of MPC/alginate or early BAC/alginate cultures that could be used to guide the selection of loading parameters. Compression peak amplitude, compression static offset, and duration of the load-radiolabel period were based upon protocols with BAC/agarose cultures and with mature BAC/alginate cultures [24, 41, 62, 189].

For dynamic loading, gel disks were subjected to oscillatory uniaxial unconfined compression using a compression device recently developed by our collaborators for loading studies with various tissue explants and cell-seeded constructs. The compression device consisted of bioreactor cassettes, the loading frame, a digital control unit, and a power supply (Figure 17). Each polysulfone bioreactor cassette had a base containing 8 individual cylindrical wells into which the gels were placed and had a top with a cylindrical, solid, stainless steel platen located above the position of each well. The cassettes were slid into a central shallow groove in the stationary base plate of the loading frame, and a total of four cassettes could be accommodated in the loading frame. The position of each cassette top, and thus the amount of compressive deformation applied to the gels, was determined by the position of the upper plate of the loading frame to which each top was mounted, and this upper plate was capable of controlled, uniaxial motion.

A square rail linear table was attached to the base plate of the loading frame. The servo motor was driven by a servo drive. The upper plate of the loading frame was bracketed to the front face of the linear table. Vertical motion was confirmed with a linear encoder affixed external to the linear table. Programming software was used to apply a sinusoidal motion profile at the specified frequency.

For static loading, gel disks were subjected to a static uniaxial unconfined compression using the same polysulfone reactors used for dynamic loading or using a polycarbonate bioreactor (described previously in Chapter 4 methods). For each type of reactor, the position of the lid of the reactor, and thus the amount of compressive deformation applied to the gels, was determined by the height of machined spacer bars. The spacer bars fit into aligned grooves in the base and lid of the bioreactor. Preliminary testing verified that both MPC and BAC cultures held at 15% static compression for 20 hours had ^{3}H-proline and ^{35}S-sulfate incorporation rates comparable to cultures held at 0% static compression (data not shown), consistent with a study demonstrating no response to static compression in early BAC/agarose cultures [24].

Experimental Design

A series of three studies was conducted to evaluate biosynthesis of MPC and BAC cultures with application of continuous oscillatory compressive loading (Table 7).

Study 1: Preliminary Loading Frequency Comparison

The goal of the initial study was to identify a loading frequency that would stimulate proteoglycan synthesis in day 1 BAC/alginate cultures. A pair of experiments was conducted with day 1 BAC cultures in which two loading frequencies were evaluated in

each experiment (1.0 Hz and 0.33 Hz; 0.33 Hz and 0.1 Hz). Due to limited donor cell numbers, different BAC donors were used in the two experiments.

Study 2: Influences of Cell Type and Culture Time on Mechanoresponsiveness

With completion of the first study, the 0.1 Hz loading frequency was selected as an appropriate loading frequency based upon the observed stimulation of proteoglycan synthesis in the day 1 BAC cultures. Subsequently, a study was conducted at 0.1 Hz to evaluate biosynthesis of MPCs and BACs with oscillatory loading. Loading with radiolabel was initiated on days 1, 4, and 7. Two experiments were conducted at separate times. The same MPC donor but different BAC donors were used in the two experiments. Additionally, in the second loading experiment, media supplementation was an additional experimental variable evaluated for day 1 MPCs, with the response of MPCs cultured in the absence of TGF-β1 compared to the response of MPCs cultured in the standard chondrogenic media containing 10ng/ml TGF-β1.

Study 3: Influence of Loading Frequency on Mechanoresponsiveness

Based in part upon observations in the initial loading study, two experiments were conducted to further evaluate how varying the loading frequency would influence biosynthesis during oscillatory compressive loading. Given the noted influence of loading frequency on day 1 BAC biosynthesis, it was of interest to determine whether altering the loading frequency would modulate day 1 MPC biosynthesis. Therefore, an experiment was conducted to evaluate biosynthesis with oscillatory loading at higher frequencies (0.33 Hz and 1.0 Hz) and compared the response of day 1 MPC cultures supplemented with TGF-β1 to cultures lacking TGF-β1 supplementation (1 donor; 1 experiment). As the loading frequency was found to influence both day 1 BAC and MPC

biosynthesis, it was of interest to determine whether sensitivity to loading frequency was maintained with additional time in culture. Therefore, an experiment was conducted to evaluate biosynthesis with oscillatory loading at higher frequencies (0.33 Hz and 1.0 Hz) for day 7 MPC and BAC cultures (1 donor per cell type; 1 experiment).

Biochemical Analyses

Gels were lyophilized, weighed, resuspended in papain digest solution (150mM sodium phosphate dibasic, 5mM Na_2EDTA, 5mM n-acetyl-L-cysteine pH 6.5 containing ~1.7 units papain per sample), and incubated at 60ºC for 24 hours. For radiolabel incorporation measurements, 100µl of the sample digest was added to 2 ml Ecolume in a scintillation vial. Raw counts in CPM were obtained for each sample using a liquid scintillation counter. ^{3}H and ^{35}S activity were calculated from the raw counts based upon standard curves prepared from serial dilutions of the original radiolabeled media. The activities were then converted into ^{3}H-proline and ^{35}S-sulfate incorporation rates. Double-stranded DNA content was determined using the PicoGreen assay and quantified against a lambda DNA standard curve. A 1:20 sample dilution was prepared in base buffer. 100µl sample dilution was mixed with 100µl PicoGreen reagent (prepared in 1X TE buffer according to the vendor instructions). The mixture was incubated for 10 minutes and then measured on a Perkin-Elmer HTS 7000plus plate reader (excitation at 485nm; emission at 535nm). For selected samples, sulfated glycosaminglycan (sGAG) content was determined using a modified DMMB assay appropriate for use with alginate-containing samples [159]. sGAG content in experimental samples was quantified against a chondroitin-4-sulfate standard from bovine cartilage. Alginate, at a concentration matching that in the sample dilutions, was included in the buffer used to create the

chondroitin-4-sulfate standard curve to account for any interaction between the dye and alginate. Sample dilutions were prepared as necessary in the appropriate base digest buffer. 200µl dye (~21mg/L dye in 29mM sodium formate, pH 1.5 adjusted with formic acid) was added to 20µl sample dilution in a 96-well plate and then measured immediately at 525nm on a Bio-Tek PowerWave X340 plate reader.

Mechanical Testing of Alginate Cultures

To characterize the behavior of alginate cultures subjected to oscillatory compressive loading, mechanical testing was performed on MPC-seeded gel samples prepared and cultured identically to those used in loading experiments. For each test, a gel sample was placed into a cylindrical sample holder, which was screwed directly into the top surface of a 1.1lbf load cell. The load cell was affixed to the testing frame of the ELectroForce 3200 testing system (EnduraTEC, Minnetonka, MN). The sample holder was filled with ITS^{+}-containing culture media. A solid stainless steel platen was used to apply controlled deformation to the upper face of the gel sample, resulting in uniaxial, unconfined compression of the sample. The testing protocol was designed to simulate dynamic loading conditions. Initially, the sample was ramped to 10% compression, held for 30 minutes to account for the time associated with loading set-up in an actual experiment, ramped further to 15% compression and then cyclic loading (±3% deformation at the specified loading frequency) was initiated. Data were captured for the first 50 seconds of every 11 minutes for ~3.5 hours. MPC-seeded alginate gels grown to culture days 1 and 7 were evaluated. For testing on culture day 1, samples were evaluated for all three loading frequencies used in the loading experiments (n=1-2/frequency). For loading on day 7, samples were evaluated at frequencies of 0.1 and 1 Hz (n=2/frequency). The

dynamic compressive modulus was estimated from the slope of the stress-strain curve corresponding to the tenth compression cycle ranging from 15% to 18% deformation for 1 Hz loading on day 1.

Statistical Analyses

Data were analyzed using a general linear ANOVA model to evaluate the effect of load (or culture day in selected control, no-load analyses) on the biochemical parameter of interest for each cell type and culture day (significance at $p<0.05$), with Tukey's test utilized for post-hoc analysis where appropriate. For the pair of experiments conducted at 0.1 Hz, the ANOVA model included experiment and load for each cell type and culture day. While the data were presented normalized to the 15% static control group for each experiment, the raw data were used for statistical analyses.

Results

Preliminary Loading Frequency Comparison

A necessary step in the development of this aim was to identify a loading protocol that would be stimulatory for early BAC/alginate cultures, similar to reports in the literature for early BAC/agarose cultures. Loading frequency was the initial loading parameter evaluated. For the selected deformational protocol (15±3% compression), day 1 BAC cultures were loaded at 3 frequency levels and the response to dynamic loading was found to be frequency-dependent (Figure 18). Oscillatory loading at 1.0 Hz, the highest frequency evaluated, decreased both ^{35}S-sulfate incorporation (16%; $p=0.003$) and ^{3}H-proline incorporation (13%; $p<0.05$) compared to the 15% static group. Decreasing the loading frequency to 0.33 Hz led to a lack of response in the BAC cultures. In each experiment, there were no differences in ^{35}S-sulfate or ^{3}H-proline

incorporation between oscillatory loading at 0.33 Hz and the 15% static group. Further reducing the loading frequency to 0.1 Hz, the lowest frequency evaluated, led to a marked change in the response of the BAC cultures. Oscillatory loading at 0.1 Hz increased both ^{35}S-sulfate incorporation (17%; $p=0.001$) and ^{3}H-proline incorporation (19%; $p<0.001$) compared to the 15% static group. DNA content was comparable across all loading groups for each experiment conducted (data not shown).

Influences of Cell Type and Culture Time on Mechanoresponsiveness

With validation of a loading protocol that was stimulatory for day 1 BAC/alginate cultures, an oscillatory loading study was subsequently conducted at 0.1 Hz to assess changes in biosynthesis in MPC and BAC cultures upon application of loading at multiple times (days 1, 4, and 7) during the first week of culture. There were notable differences in the response to oscillatory loading for the two cell types at the earlier culture times (Figure 19). In BAC cultures, oscillatory loading increased ^{3}H-proline incorporation compared to the 15% static group at day 1 (14%; $p=0.001$), day 4 (9%; $p<0.005$), and day 7 (7%; $p<0.03$) and also increased ^{35}S-sulfate incorporation compared to the 15% static group at day 1 (8%; $p<0.05$) and day 4 (8%; $p<0.01$).

In contrast to the BAC cultures, there were no differences in ^{35}S-sulfate or ^{3}H-proline incorporation between the oscillatory loading group and the 15% static group for MPC cultures at day 1 or day 4. However, with additional time in culture preceding loading initiation, the MPC cultures exhibited an alteration in biosynthesis with oscillatory loading. In the day 7 MPC cultures, oscillatory loading increased both ^{3}H-proline incorporation (6%; $p<0.02$) and ^{35}S-sulfate incorporation (8%; $p<0.01$) compared to the 15% static group. No differences were found in DNA content between any of the loading

groups for each combination of cell type and culture time evaluated with the exception of day 1 MPC cultures, which were found to be inhibited with oscillatory loading compared to 15% static compression (5%; p=0.03; data not shown). Additionally, the influence of media supplementation on the response to oscillatory loading was evaluated for day 1 MPC cultures, with the response of MPCs cultured in the absence of TGF-β1 compared to the response of MPCs cultured in the standard chondrogenic media containing 10ng/ml TGF-β1. No differences in ^{35}S-sulfate or ^{3}H-proline incorporation were noted between the oscillatory loading group and the 15% static group for either media formulation (Table 8).

In evaluating the baseline activity of control MPC and BAC cultures, changes in biosynthesis and sGAG/DNA accumulation were observed during the first week of culture (Table 9). ^{35}S-sulfate incorporation, ^{3}H-proline incorporation, and sGAG/DNA all increased from day 1 to day 7 in the MPC cultures ($p<0.001$). For the BAC cultures, sGAG/DNA increased from day 1 to day 7 ($p<0.001$), while ^{35}S-sulfate incorporation and ^{3}H-proline incorporation initially increased from day 1 to day 4 ($p<0.005$), then ^{35}S-sulfate incorporation decreased to an intermediate value ($p<0.001$) but ^{3}H-proline incorporation did not change from day 4 to day 7.

Influence of Loading Frequency on Mechanoresponsiveness

Observations from the initial loading study prompted further experimentation to evaluate how oscillatory loading at higher frequencies (0.33 Hz and 1.0 Hz) would influence biosynthesis during oscillatory compressive loading. Given the noted influence of loading frequency on day 1 BAC biosynthesis, an experiment was conducted to determine whether day 1 MPCs also exhibited sensitivity to loading frequency. In this

experiment, the response of MPC cultures supplemented with TGF-β1 was compared to the response of cultures lacking TGF-β1 supplementation. There were no differences in ^{35}S-sulfate or ^{3}H-proline incorporation between the oscillatory loading groups at the two loading frequencies evaluated and the 15% static group for either media formulation, with one noted exception (Table 10). Oscillatory loading at 1.0 Hz inhibited ^{35}S-sulfate incorporation compared to the 15% static group (16%; $p<0.002$) for MPCs grown in media containing TGF-β1, but did not alter ^{35}S-sulfate incorporation compared to the 15% static group for MPCs grown in media lacking TGF-β1. DNA content was comparable between the loading groups for each media formulation (DNA data not shown).

Since loading frequency was found to influence both day 1 BAC and MPC biosynthesis, an experiment was conducted with day 7 cultures to determine if sensitivity to loading frequency was maintained with additional time in culture. Oscillatory compressive loading modulated radiolabel incorporation rates in both BAC and MPC cultures, and the response across the two cell types was markedly different. However, the two frequencies evaluated (1.0 and 0.33 Hz) elicited similar effects for each cell type (Figure 20). In BAC cultures, ^{3}H-proline and ^{35}S-sulfate incorporation were stimulated in the 1.0 Hz loading group compared to the 15% static group (^{3}H-proline at 19%; $p<0.001$; ^{35}S-sulfate at 10%; $p<0.02$) and in the 0.33 Hz loading group compared to the 15% static group (^{3}H-proline at 16%; $p<0.003$; ^{35}S-sulfate at 10%; $p<0.02$). DNA was also found to be inhibited in the 1.0 Hz loading group compared to the 15% static group (7%; $p<0.02$; data not shown). In MPC cultures, ^{35}S-sulfate incorporation was inhibited in the 1.0 Hz loading group compared to the 15% static group (14%; $p<0.001$) and in the 0.33 Hz

loading group compared to the 15% static group (11%; $p<0.008$), but ^{3}H-proline incorporation and DNA content were comparable between the loading groups (DNA data not shown).

Mechanical Testing of MPC-Seeded Alginate Cultures

Mechanical testing was performed to investigate the behavior of MPC-seeded alginate cultures subjected to oscillatory compressive loading, and gels were evaluated at days 1 and 7. As a baseline comparison to other hydrogel culture systems, the dynamic compressive modulus of the cell-seeded 2% alginate gels at day 1 was estimated to be 69kPa. Hysteresis was observed in the stress-strain curves for the cell-seeded alginate gels (Figure 21; data shown at 0.1 Hz), indicating that nonlinear behavior occurred with oscillatory loading due to the viscoelastic nature of the alginate. Stress relaxation was observed in all samples, with a rapid drop in the peak compressive loads during the first ~10 minutes of loading, a further drop over the next ~30-60 minutes, and then a more gradual decline throughout the remainder of testing (data not shown).

A primary reason for conducting the mechanical testing was to evaluate whether lift-off, the loss of contact between the loading platen and the gel face, occurred during oscillatory compressive loading. A notable static offset was incorporated into the oscillatory loading protocol to compensate for the viscoelastic nature of the alginate gel and to minimize the potential for lift-off. Traces of position versus time and load versus time (Figures 22-23) were compared for assessment of lift-off. At loading initiation, load traces showed compressive loads throughout, indicating that contact was maintained between the platen and the gel surface. It should be noted that the load curves were not

completely symmetric. The peak corresponding to the highest platen position was broader than the peak corresponding to lowest platen position.

After loading for three hours, the load traces showed a zero (or positive) load level corresponding to the highest platen position for both the day 1 and day 7 gels, although the roughly sinusoidal shape of the load-time curve was maintained. The minimum compressive load was found to become more positive with further time in culture (data not shown), suggesting the presence of an artifact in the load cell readings. The artifact could have resulted from incorrect taring of the load cell and/or drift in the load cell during testing, although the source of the artifact could not be determined during testing of multiple samples over several days. Therefore, the load values were considered to be inaccurate and the gels were likely not experiencing actual zero loads during mechanical testing. Furthermore, given that there was no flattened or linear appearance in the load curve corresponding to the region of the highest platen position, the data suggest that contact was maintained between the platen and the gel surface. Overall, mechanical testing to replicate the specific protocol used in the oscillatory loading experiments indicated that stress relaxation was significant but lift-off was minimal during oscillatory loading of MPC-seeded alginate gels at culture days 1 and 7. Additionally, no major differences were noted in the behavior of the alginate gels over the frequency range evaluated.

Discussion

Orthopaedic tissues such as cartilage and bone experience dynamic and complex mechanical stimulation under normal loading. Mechanical stimulation also plays a role in orthopaedic tissue development and repair. MPCs have been identified as a potential

cell source for orthopaedic tissue engineering strategies, which substantiates the need for extensive exploration into the mechanoresponsiveness of these cells. Determining how progenitor populations respond to types of loading known to modulate differentiated cells is one critical area of investigation. This work evaluated biosynthesis during continuous oscillatory compressive loading of MPC/alginate cultures utilizing a loading protocol shown to enhance biosynthesis of articular chondrocytes in hydrogel suspension.

MPCs supplemented with TGF-β1 to support chondrogenic differentiation were demonstrated to alter biosynthesis in a culture time-dependent and frequency-dependent manner upon application of oscillatory unconfined compressive loading. MPCs showed a lack of response to 0.1 Hz oscillatory loading at culture days 1 and 4, but were found to respond to oscillatory loading in a stimulatory manner for both ^{35}S-sulfate and ^{3}H-proline incorporation at culture day 7. The additional time in culture preceding load initiation supported increases in proteoglycan synthesis and sGAG content, indicative of the progression of chondrogenic differentiation. Chondrogenic differentiation could result in cellular changes that support transduction of mechanical signals resulting from oscillatory compression, such as changes in cytoskeletal components, membrane channels, or cell-surface proteins. However, proteoglycan accumulation in the day 7 cultures resulting from the extended time preceding loading initiation could also support specific interactions between MPCs and extracellular matrix components, such as hyaluronan. Human MPCs have been shown to express CD44, the hyaluronan receptor, and CD29, the β1 integrin subunit [113]. Integrins have been previously demonstrated to play a role in chondrocyte mechanotransduction [206]. The response of the day 7 MPC cultures to oscillatory loading could be related in part to these matrix interactions. Importantly,

modulation of biosynthesis in day 7 cultures was demonstrated to depend on the loading frequency. Inhibition of ^{35}S-sulfate incorporation occurred with oscillatory loading at 1.0 Hz, in contrast to the stimulatory response observed with loading at 0.1 Hz.

In contrast to our work, a preliminary radiolabel-compression study evaluating human adipose-derived adult stem cells in agarose suspension with chondrogenic media observed inhibition of biosynthesis with oscillatory loading at 0.1 Hz [207]. In that study, continuous cyclic mechanical compression beginning on culture days 4 or 7 inhibited ^{3}H-proline incorporation and decreased the DNA content of the cultures, while day 4 loading also inhibited ^{35}S-sulfate incorporation. The dissimilar results noted between our work and this study, particularly at the day 7 culture time, could be related to possible differences between the two cell types or to experimental differences, including the gel material (alginate versus agarose) and the load duration (20 hours versus 48 hours).

The influence of TGF-β1 supplementation on MPC biosynthesis during oscillatory compressive loading was directly assessed by comparing the response of MPC cultures supplemented with TGF-β1 to MPC cultures lacking TGF-β1 supplementation for loading on day 1. The results of altering TGF-β1 supplementation clarified that day 1 MPCs were unresponsive to oscillatory loading at 0.1 Hz whether cultured in the presence or absence of TGF-β1. However, an interesting difference was noted with loading at 1.0 Hz. Cultures grown in media lacking TGF-β1 were insensitive to oscillatory compressive loading, in contrast to the inhibitory response demonstrated by MPCs grown in media with TGF-β1. This may indicate that the undifferentiated MPCs lack the ability to transduce mechanical signals from oscillatory compressive loading in

alginate suspension, while TGF-β1 stimulation could result in rapid cellular changes enabling transduction of mechanical signals in the day 1 cultures, albeit transduction resulting in inhibition of proteoglycan synthesis.

While the experiments in this chapter focused on MPC biosynthesis during oscillatory compressive loading, it was necessary to evaluate BAC biosynthesis during oscillatory loading to help identify appropriate loading parameters given the lack of information regarding loading of early BAC/alginate cultures. It was found that BACs modulated proteoglycan and protein synthesis during oscillatory compressive loading at early culture times ranging from 1 to 7 days. Changes in biosynthesis were strongly influenced by loading frequency at day 1. At the lowest frequency evaluated (0.1 Hz), oscillatory compressive loading stimulated radiolabel incorporation rates in day 1 cultures, but increasing the loading frequency by a single order of magnitude resulted in an inhibition of radiolabel incorporation rates. At day 7, modulation of ^{3}H-proline incorporation was independent of loading frequency, while modulation of ^{35}S-sulfate incorporation showed a limited dependence on frequency.

Previous reports on loading of early BAC/agarose cultures have been contradictory regarding frequency influences. Buschmann et al. showed that continuous oscillatory compression stimulated ^{35}S-sulfate and ^{3}H-proline incorporation for BACs in agarose suspension (day 2-5 cultures) and the response was found to be comparable across loading frequencies ranging from 1.0 to 0.01 Hz [24]. Lee and Bader showed that continuous oscillatory compression of day 1 BAC/agarose cultures influenced sGAG accumulation in a frequency-dependent manner ranging from inhibitory at 0.3 Hz to stimulatory at 1.0 Hz to no change at 3.0 Hz, while ^{3}H-proline incorporation was

inhibited at all frequencies [62]. Differences in the frequency response between our work and these studies could be related in part to differences in the gel material used, with agarose being a stiffer material than alginate [15]. Additionally, the duration of continuous loading was different across the studies and the compression amplitude used in the Lee and Bader study was more than double the amplitude level used in both our work and the Buschmann study.

Comparing changes in biosynthesis upon application of oscillatory loading for the MPC and BAC cultures at different frequency levels and different culture times revealed some interesting observations. Changes in proteoglycan synthesis during oscillatory compressive loading were similar at 1.0 Hz but different at 0.1 Hz in the two cell types at day 1. Since mammalian cells cannot specifically interact with the surrounding alginate [193] and extracellular matrix accumulation would be limited at this early culture time, the two cell types were likely subjected to similar changes in the local environment with oscillatory compressive loading. One possible explanation for the different cell responses is that distinct signal transduction pathways are being utilized to modulate ^{35}S-sulfate incorporation for the two loading frequencies. The MPCs would appear to have the appropriate cellular machinery to respond to the higher frequency signal but not to the lower frequency signal, while BACs are responsive to both signals. It seems quite plausible that BACs freshly isolated from tissue and culture-expanded prechondrogenic MPCs could have different levels of cytoskeletal organization and membrane-associated proteins that might result in distinct signal transducing capabilities. At day 7, proteoglycan synthesis was stimulated in BAC cultures but inhibited in MPC cultures with 1.0 Hz oscillatory loading, while proteoglycan synthesis was stimulated in MPC

cultures with 0.1 Hz oscillatory loading. This frequency difference may suggest that the mechanisms supporting ^{35}S-sulfate incorporation increases at day 7 are distinct in the two cell types. Interactions between cells and the developing pericellular matrix may be more substantial for BACs than MPCs at day 7, for example, given the more rapid sGAG accumulation in BAC cultures during the first week of culture.

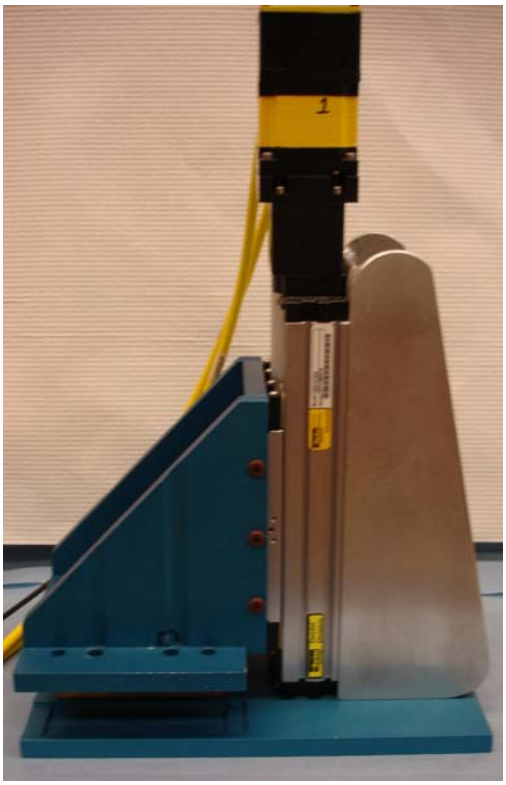
Oscillatory unconfined compressive loading of the alginate cultures will produce a number of changes in the local environment around the cells which could contribute to the modulation of biosynthesis observed in this work, including changes in fluid pressure and fluid flow. With unconfined compression, fluid pressure and fluid flow depend on radial position within the gel [208]. Maximum fluid pressure occurs in the center of the gel and minimum pressure occurs at the gel periphery. Conversely, fluid flow velocity is minimal in the gel center and highest at the periphery. The distribution of fluid pressures and fluid flow velocities within the gel will depend on loading frequency. The measured peak stress can be used to approximate the peak fluid pressure resulting from oscillatory loading [209]. The peak stress resulting from oscillatory loading of the MPC/alginate cultures was determined to be <5 kPa by mechanical testing. Since this peak stress is several orders of magnitude lower than the hydrostatic pressures shown to stimulate biosynthesis in chondrocyte-seeded agarose gels [210], it seems unlikely that fluid pressurization contributed to the loading response in these experiments. Fluid flow could contribute to enhanced transport. However, a recent modeling study of oscillatory unconfined compression in agarose gels found minimal fluid flow in the gel with loading [191].

Matrix-independent cell deformation and/or cell-matrix interactions will also be present with oscillatory compressive loading, depending on the amount of matrix present around the cells. With unconfined compression of cylindrical gels, there will be compressive strains applied along the loading axis and tensile strains applied along the radial axis. It has been demonstrated that gross 20% uniaxial compression of day 1 chondrocyte/alginate cultures results in cell and nucleus deformation [194]. Stretch-activated ion channels supporting calcium influx have been identified in chondrocyte cultures [65], and membrane stretch associated with cellular deformation could lead to channel activation. However, the production of a local pericellular matrix may notably alter the degree of deformation experienced by cells in hydrogels subjected to compression. One study found that the extracellular matrix produced in culture after 3-6 days protected isolated BACs in agarose from deforming under gross 20% compressive strain [197]. It is not known if the degree of matrix accumulation in the alginate gels at day 7 would reduce compression-induced deformation of either BAC or MPC cultures similar to that observed in agarose cultures. The loading frequency differences observed in these experiments could result from a strain rate-dependent signaling mechanism, which could potentially be mediated by cell deformation or cell-matrix interactions.

Very limited knowledge is available regarding mechanisms responsible for the modulation of ^{35}S-sulfate and ^{3}H-proline incorporation during compressive loading of cartilage explants or isolated chondrocytes. Uniaxial compression of BAC/agarose cultures and fluid flow over chondrocyte monolayers have both been shown to increase intracellular calcium and may involve calcium influx through various types of ion channels [211, 212]. Intracellular calcium is involved in many signaling pathways,

although direct links to proteoglycan and protein synthesis appear to be lacking. Lowering the pH of the local environment around chondrocytes, such as occurs with static compression of cartilage explants, has been directly linked to inhibition of biosynthetic rates [213]. However, this mechanism would not be present in oscillatory compressive loading of cell-seeded gels at amplitudes in the range of 3%. In a much different system without loading influences, protein synthesis was shown to be sensitive to intracellular potassium levels in oocytes [214]. If this observation could be applied broadly to intracellular ion levels, it would suggest a mechanism for protein synthesis modulation applicable to compressive loading of chondrocytes and MPCs. While loading influences on chondrocyte biosynthesis have been described for many different types of loading and cell culture formats, much remains to be learned about the underlying transduction events supporting these changes.

In conclusion, this work has demonstrated that MPC cultures undergoing chondrogenic differentiation respond to oscillatory unconfined compressive loading in a culture time-dependent manner. For a loading protocol that stimulated biosynthesis in chondrocyte cultures, oscillatory loading stimulated proteoglycan and protein synthesis in day 7 MPC cultures, but the stimulatory response did not occur in earlier MPC cultures. For both MPC and BAC cultures, complex interactions were observed between culture time, loading frequency, and the modulation of biosynthesis, which may reflect inherent characteristics of the alginate culture system used in this work.



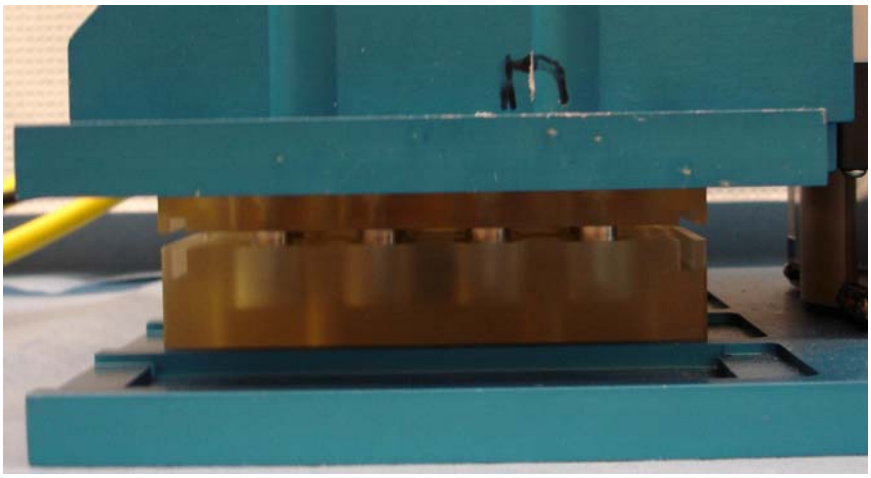


Figure 17: Dynamic Loading System for Radiolabel-Compression Experiments. Top: Compression device (i.e. dynamic loading system) used to apply controlled deformation to gels situated within the bioreactor cassette via stainless steel platens located on the bottom surface of the bioreactor lid. The bioreactor lid is mounted to the upper plate of the loading frame, and this upper plate is bracketed to the front face of a linear table. Bottom: Close view of bioreactor cassettes attached to the loading frame.

Table 7: Summary of Studies Evaluating MPC and BAC Biosynthesis During Oscillitory Loading. For studies 2 and 3, media supplementation (± TGF-β1) was a secondary experimental variable that was evaluated for day 1 MPC cultures.

	Experimental Variable *(Experimental Constant)*	Levels Evaluated	Cell Type
Study 1	Loading Frequency *(Day 1 Loading)*	1.0, 0.33, or 0.1 Hz	BAC
Study 2	Loading Day *(0.1 Hz Loading Frequency)*	Day 1, Day 4, or Day 7	MPC & BAC
Study 3	Loading Frequency *(Day 1 & Day 7 Loading)*	1.0 or 0.33 Hz	MPC & BAC

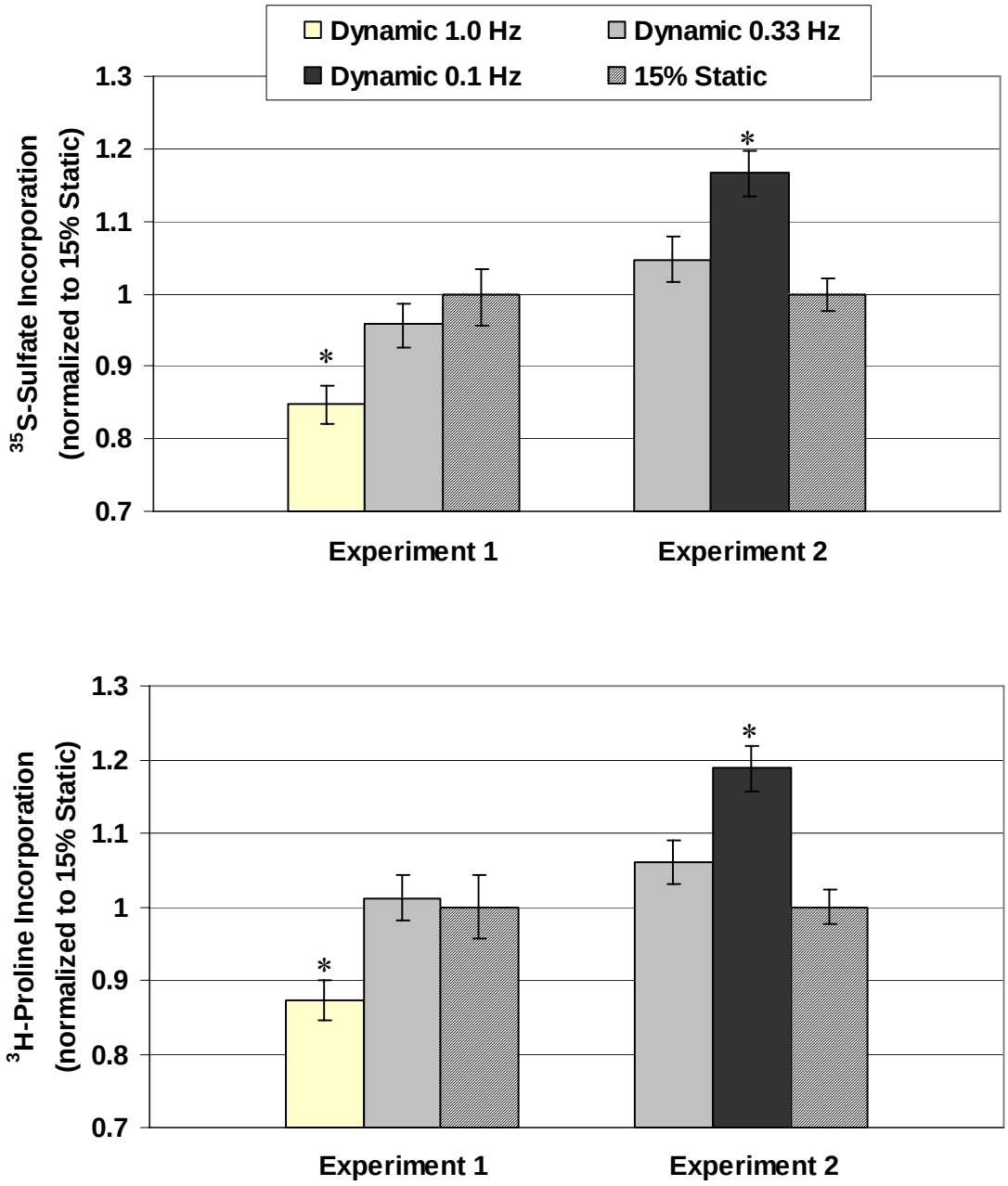


Figure 18: Loading Frequency Comparison for Oscillatory Compressive Loading of BAC/Alginate Day 1 Cultures. Biosynthesis during loading was evaluated in dynamically loaded gels (15 ± 3% compression) for oscillatory frequencies of 1.0 Hz, 0.33 Hz, or 0.1 Hz and was compared to biosynthesis in control gels held at 15% static compression. For each loading frequency evaluated, the dynamically loaded data was normalized to its paired 15% static data. Values are mean ± SEM (n=8 gels/loading group/experiment). * denotes significantly different vs. 15% static.

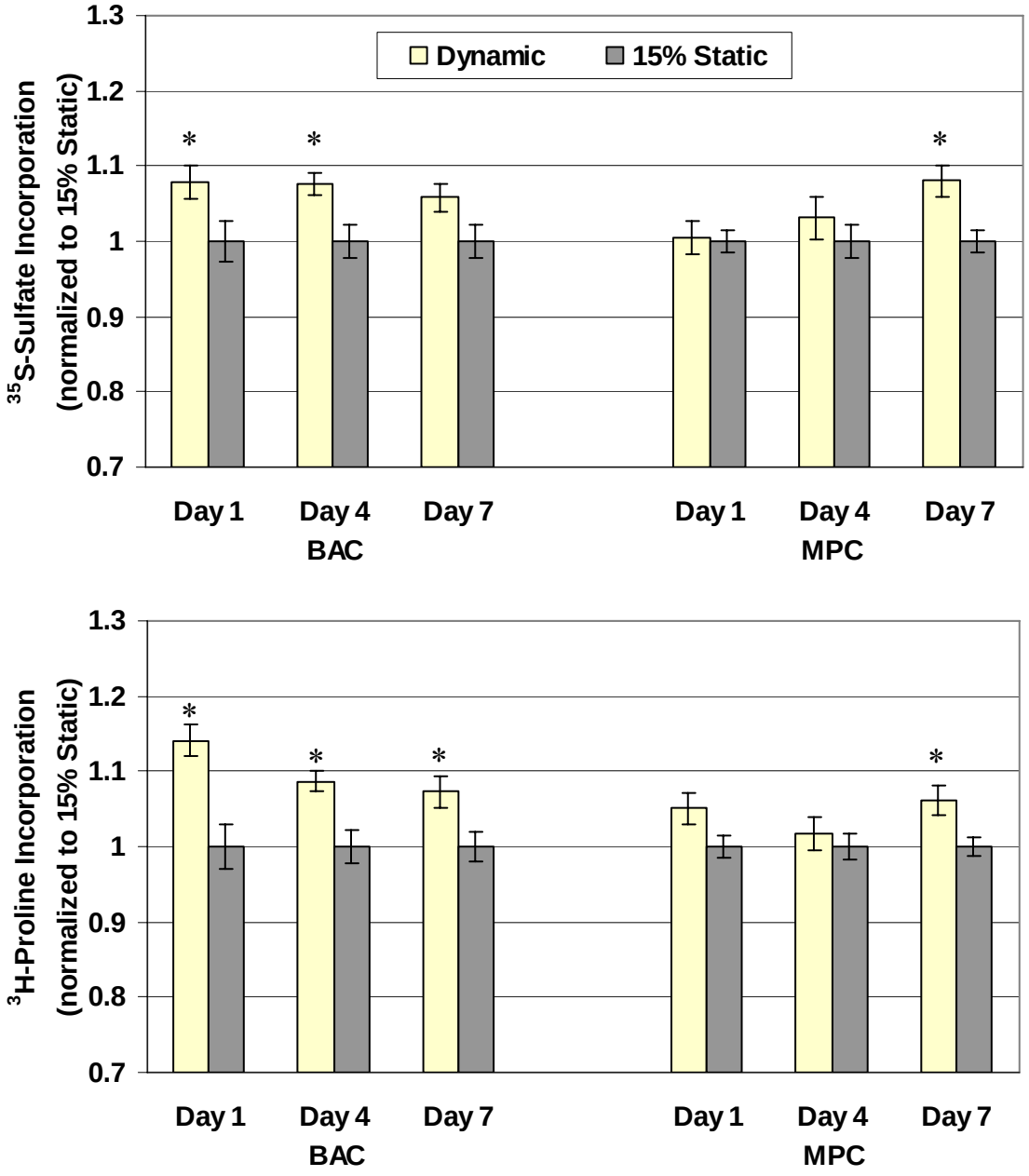


Figure 19: Cell Type and Culture Time Effects on Mechanoresponsiveness. Biosynthesis during oscillatory compressive loading was evaluated on days 1, 4, and 7 in both BAC and MPC cultures. MPCs were grown in chondrogenic media (media with ITS^{+} and TGF-β1) and BACs were grown in media with 10% FBS. Dynamically loaded gels (15 ± 3% compression; 0.1 Hz) were compared to control gels held at 15% static compression. Each dynamically loaded data set was normalized to the paired 15% static data set. Values are mean ± SEM (n=7-8 gels/loading group/experiment; 2 experiments). * denotes significantly different vs. 15% static.

Table 8: Effects of TGF-β1 Supplementation on Biosynthesis during Loading for Day 1 MPC Cultures. Starting on Day 0, MPC gels were cultured in the absence of TGF-β1 (-TGF-β1) or were supplemented with TGF-β1 at 10ng/ml throughout (+TGF-β1). Dynamically loaded gels (15 ± 3% compression; 0.1 Hz) were compared to control gels held at 15% static compression for loading on culture day 1. Values are mean ± SEM (n=8 gels/loading group; 1 experiment).

Parameter (normalized to 15% Static)	^{35}S-Sulfate Incorporation	^{3}H-Proline Incorporation
+TGF-β1		
Dynamic	1.036 ± 0.036	1.049 ± 0.035
15% Static	1.000 ± 0.024	1.000 ± 0.020
-TGF-β1		
Dynamic	0.931 ± 0.033	0.949 ± 0.028
15% Static	1.000 ± 0.048	1.000 ± 0.042

Table 9: Biosynthesis and Matrix Accumulation for BAC and MPC Cultures. MPCs were grown in chondrogenic media (media with ITS^{+} and TGF-β1) and BACs were grown in media with 10% FBS. Cultures were grown to days 1, 4, and 7. Samples are from the 15% static compression group of the second loading experiment shown in Figure 19 (mean±SEM; n=8gels/day/cell type). ***** denotes significantly different versus Day 1 for the designated cell type. ‡ denotes significantly different versus Day 4 for the designated cell type.

MPC	sGAG/DNA (μg/μg)	^{35}S-Sulfate Incorporation (pmol/ngDNA/hr)	^{3}H-Proline Incorporation (pmol/ngDNA/hr)
Day 1	1.06 ± 0.04	0.032 ± 0.001	0.229 ± 0.005
Day 4	* 5.21 ± 0.15	* 0.169 ± 0.006	* 0.320 ± 0.008
Day 7	*‡ 19.66 ± 0.69	*‡ 0.322 ± 0.009	*‡ 0.348 ± 0.008
BAC			
Day 1	6.76 ± 0.34	0.202 ± 0.009	0.161 ± 0.008
Day 4	* 24.96 ± 0.59	* 0.350 ± 0.014	* 0.200 ± 0.008
Day 7	*‡ 37.32 ± 0.99	*‡ 0.284 ± 0.010	* 0.204 ± 0.006

Table 10: Effects of TGF-β1 Supplementation on Biosynthesis during Loading for Day 1 MPC Cultures Loaded at Higher Frequencies. Starting on Day 0, MPC gels were cultured in the absence of TGF-β1 (-TGF-β1) or were supplemented with TGF-β1 at 10ng/ml throughout (+TGF-β1). Dynamically loaded gels subjected to 15 ± 3% compression at frequencies of 1.00 Hz or 0.33 Hz were compared to control gels held at 15% static compression for loading on culture day 1. Values are mean ± SEM (n=8 gels/loading group; 1 experiment). * denotes significantly different vs. 15% static.

Parameter (normalized to 15% Static)	^{35}S-Sulfate Incorporation	^{3}H-Proline Incorporation
+TGF-β1		
Dynamic 1.00Hz	*0.836 ± 0.014	0.983 ± 0.018
Dynamic 0.33Hz	0.903 ± 0.017	0.980 ± 0.024
15% Static	1.000 ± 0.044	1.000 ± 0.045
-TGF-β1		
Dynamic 1.00Hz	1.053 ± 0.048	1.118 ± 0.031
Dynamic 0.33Hz	1.050 ± 0.040	1.116 ± 0.027
15% Static	1.000 ± 0.048	1.000 ± 0.042

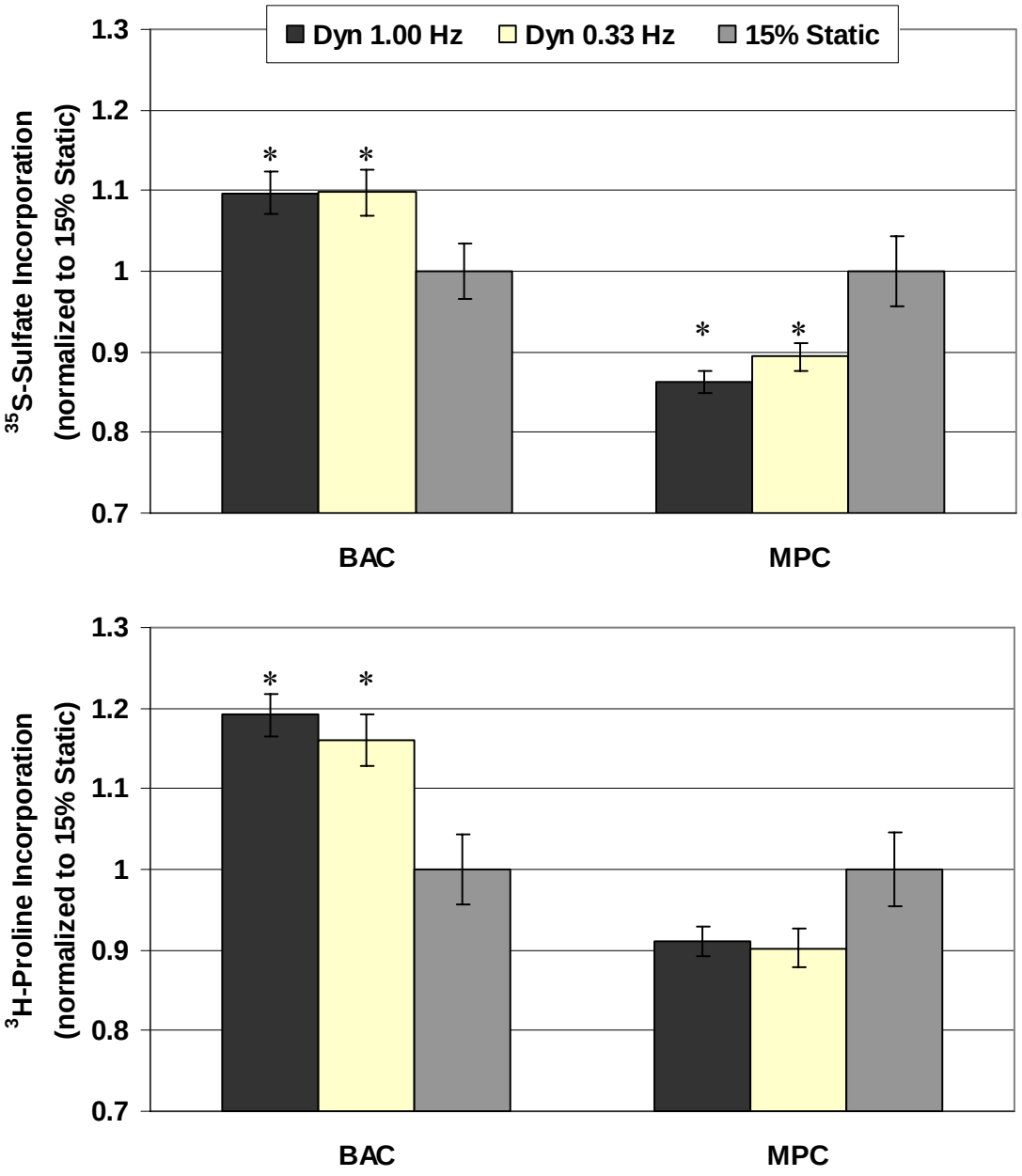


Figure 20: Evaluation of Biosynthesis during Loading for Day 7 Cultures at Higher Loading Frequencies. Dynamically loaded gels subjected to 15 ± 3% compression at frequencies of 1.00 Hz or 0.33 Hz were compared to control gels held at 15% static compression for both BAC and MPC cultures. BACs were grown in media with 10% FBS, while MPCs were grown in chondrogenic media with ITS^{+} and TGF-β1. Values are mean ± standard error and have been normalized to the 15% static control level (n=8 gels/loading group; 1 experiment). * denotes significantly different vs. 15% static.

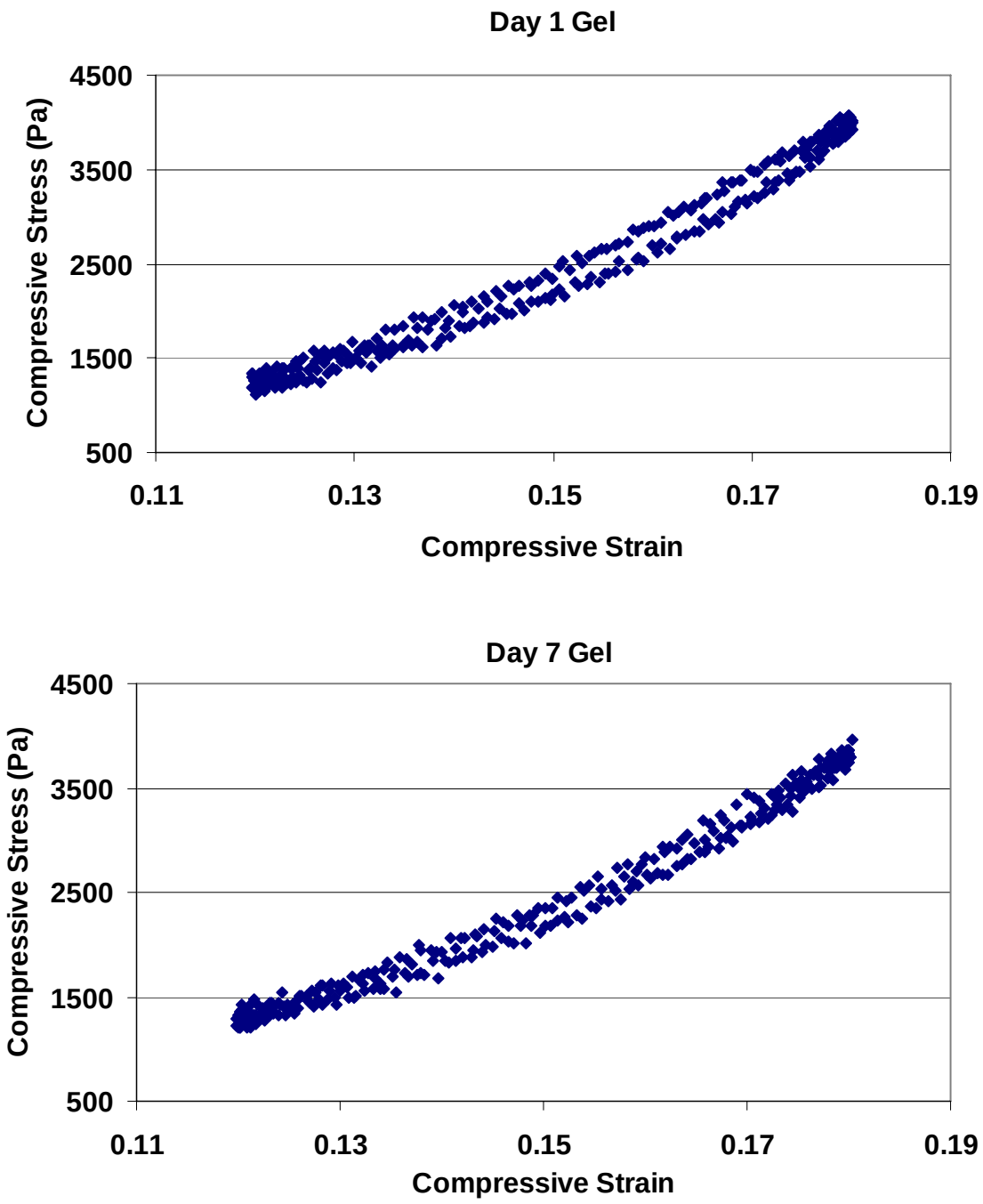


Figure 21: Stress-Strain Curve for Oscillatory Compressive Loading at 0.1 Hz. The stress-strain curve for the first loading cycle is shown for a day 1 gel (top) and a day 7 gel (bottom).

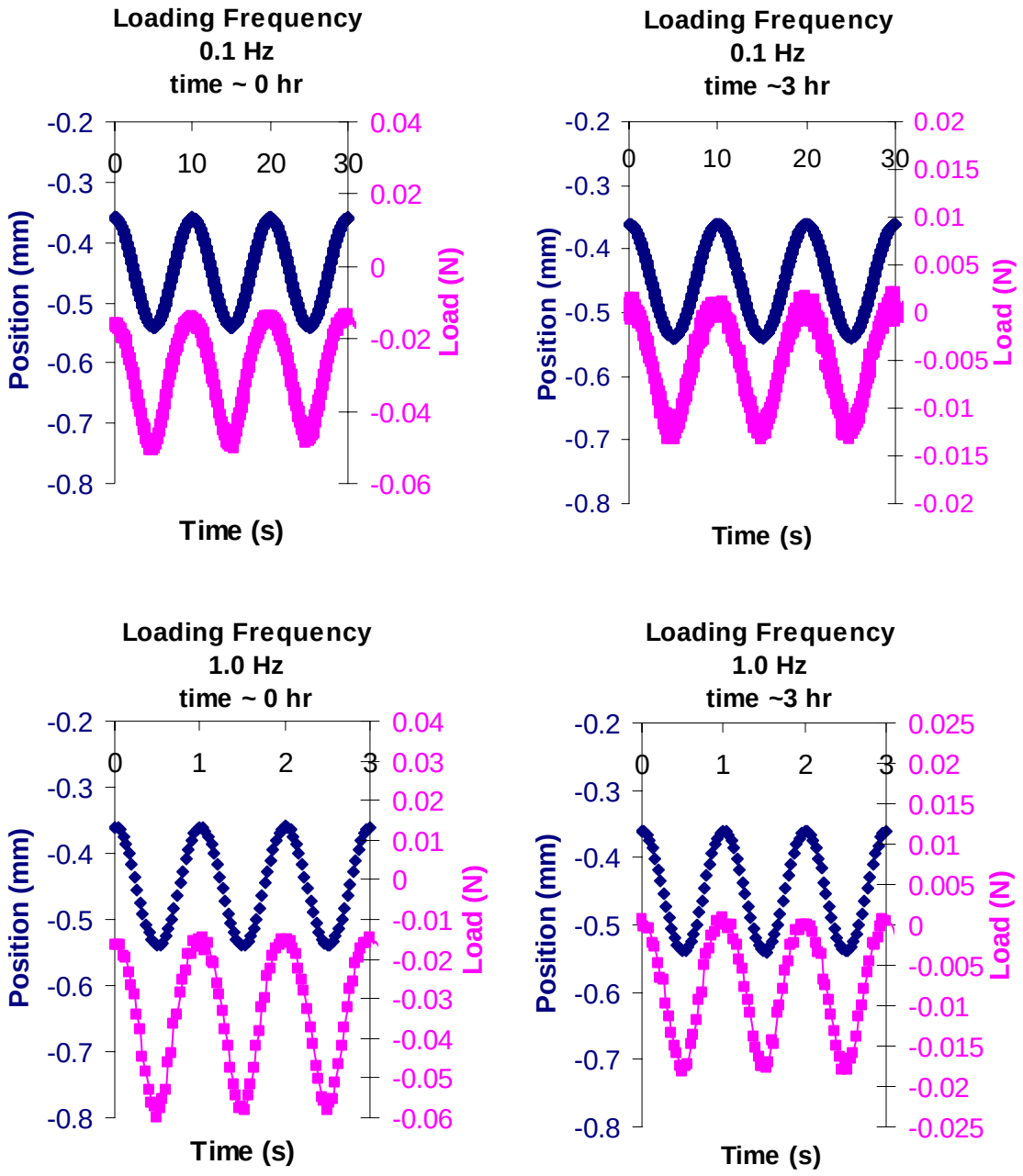


Figure 22: Comparing Load and Position Waveforms for Day 1 Gels. Oscillatory compressive loading was conducted at 1.0 Hz (top) and 0.1 Hz (bottom). Waveforms for three loading cycles are shown corresponding to loading initiation (i.e. 0 hours) and to loading 3 hours after initiation.

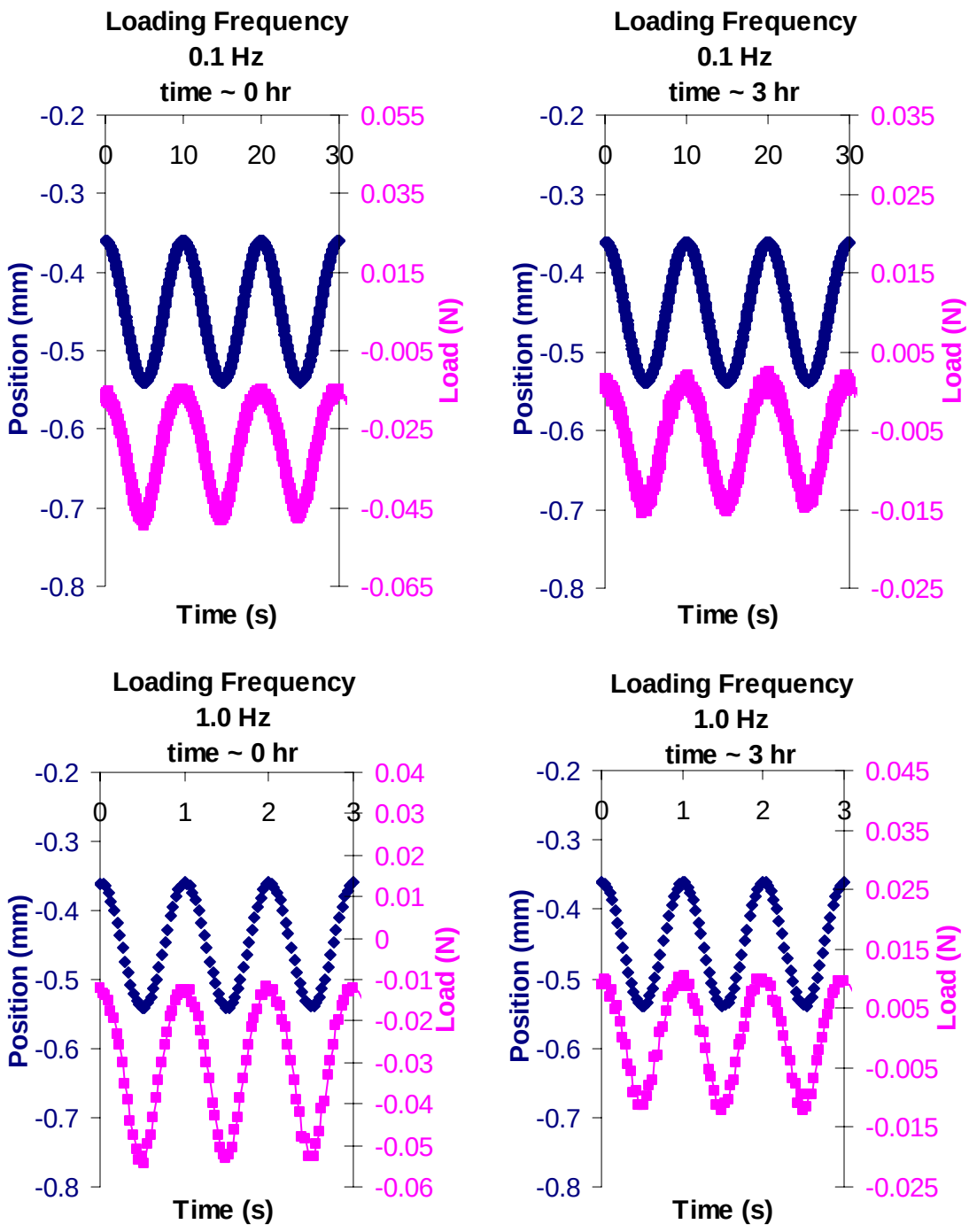


Figure 23: Comparing Load and Position Waveforms for Day 7 Gels. Oscillatory compressive loading was conducted at 1.0 Hz (top) and 0.1 Hz (bottom). Waveforms for three loading cycles are shown corresponding to loading initiation (i.e. 0 hours) and to loading 3 hours after initiation.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Bone marrow-derived mesenchymal progenitor cells (MPCs) hold much promise as an alternative cell source for cartilage tissue engineering and other cell-based cartilage repair approaches. While previous studies have established that MPCs from humans and a number of other species undergo *in vitro* chondrogenic differentiation, additional research is needed to define conditions that will enhance chondrogenic differentiation of MPCs, increase matrix production by differentiating cultures, and support development of functional tissue-engineered cartilage constructs. Mechanical loading may be an important factor in the initiation and development of chondrogenic MPC cultures. However, studies concerning the specific effects of mechanical stimulation on MPCs seeded within 3-D matrices have been limited. This thesis work evaluated the influence of dynamic mechanical stimulation on *in vitro* chondrogenic activity and biosynthesis of differentiating MPCs within hydrogel suspension.

In preparation for the loading experiments, media supplements were evaluated for promoting chondrogenic differentiation of goat MPC/alginate cultures. The conclusion of this preparatory work is that TGF-β1 is a potent supplement for stimulating chondrogenic differentiation in goat MPC/alginate cultures. Importantly, the chondrogenic response to TGF-β1 is notably enhanced by the exclusion of Dex, a factor used routinely with MPCs from other species. TGF-β1 supports the appearance of cartilage matrix components during the first week of culture. Furthermore, distinct MPC

cultures can be identified between culture days 1 and 7 based upon proteoglycan synthesis and matrix accumulation levels.

A primary conclusion of the thesis work is that oscillatory unconfined compressive loading of goat MPC/alginate cultures in chondrogenic media does not enhance the chondrogenic activity of differentiating MPCs above the level supported by chondrogenic media alone for loading applied 3 hours/day for three to six consecutive days. Furthermore, the length of the preculture period preceding loading initiation, ranging from one to seven days, does not influence the lack of effect for oscillatory loading. These conclusions have implications for the future design of mechanical preconditioning strategies to support development of cartilage constructs using MPCs.

Another primary conclusion of the thesis work is that the mechanoresponsiveness of goat MPC/alginate cultures to oscillatory unconfined compressive loading is culture time-dependent for a loading protocol shown to stimulate biosynthesis in chondrocyte cultures. Following seven days in culture with chondrogenic supplements, MPCs exhibit stimulation of proteoglycan and protein synthesis with oscillatory loading, while the stimulatory response is absent in MPCs at earlier culture times. These results help to further characterize the phenotype of differentiating MPC cultures.

A secondary conclusion of the thesis work is that the stimulation of biosynthesis during oscillatory unconfined compressive loading is dependent on the loading frequency. This conclusion is restricted to chondrogenic cultures with limited matrix accumulation, such as day 7 differentiating MPC cultures and day 1 BAC cultures. Stimulation of biosynthesis in chondrocyte cultures having more extensive matrix accumulation is relatively insensitive to loading frequency. The complex interactions

observed between culture time, loading frequency, and the modulation of biosynthesis may reflect inherent characteristics of the alginate culture system used in this work.

Comparing the two primary conclusions of the thesis work indicates differences in the MPC response to oscillatory unconfined compressive loading. The stimulation of biosynthesis during dynamic loading observed in day 7 MPC cultures, described in chapter 5, contrasts to the lack of modulation of chondrogenic activity following dynamic loading observed for all culture times, described in chapter 4. This difference in results may suggest underlying differences in the ability of MPCs to translate mechanical signals into cellular responses. The mechanotransduction pathways that would support enhancement in ^{3}H-proline and ^{35}S-sulfate incorporation during 20 hours of loading could be distinct from mechanotransduction pathways that would support sustained changes in chondrogenic activity, which would likely involve changes in gene expression leading to further changes in matrix synthesis and accumulation. However, the difference in results could also indicate that differences in the loading protocols influenced the ability of the MPCs to respond to the applied oscillatory compressive loading. The stimulation of biosynthesis was observed only at a loading frequency of 0.1 Hz, but the chondrogenic activity experiments were conducted at a loading frequency of 1.0 Hz. Also, the stimulation of biosynthesis occurred in response to 20 hours of continuous loading, while the chondrogenic activity experiments typically evaluated the response to a total of 9 hours of loading applied over three days. An additional consideration for the difference in results concerns how changes in biosynthesis are translated into detectable changes in matrix accumulation, which could be influenced by multiple timing issues including the duration of the loading period and the number of days or bouts of loading applied.

The conclusions for oscillatory compressive mechanical loading effects on MPC chondrogenic activity and biosynthesis are defined by and specific to the 3-D matrix in which the MPCs are cultured. This thesis work describes loading influences for suspended MPCs initially cultured in isolation of specific interactions with the surrounding alginate gel material, as the alginate chains are lacking appropriate adhesion sequences. However, these specific interactions could be potentially important mediators of mechanotransduction in cultures where extracellular matrix accumulation is limited. Further work is needed to more broadly understand how oscillatory compressive loading may influence MPC chondrogenic activity and biosynthesis.

Recommendations

The experiences with MPC expansion and differentiation have suggested a general recommendation for studies with MPCs, in addition to the specific suggestions noted below. In this thesis work and throughout the literature, MPCs are currently used with very little characterization of the initial population. Assessment of cell-surface marker expression, which has been used previously to a limited degree, would be one possible method for population characterization [132-134]. Using cell-surface markers to initially characterize donors and to monitor possible changes associated with *in vitro* expansion could provide insight regarding donor variability and passaging effects, issues noted in the literature to be relevant for multiple MPC species [123, 215, 216]. MPC characterization would also strengthen comparisons between studies by different investigators.

The evaluation of goat MPC chondrogenic differentiation and subsequent matrix accumulation in response to chemical stimulation would be strengthened by further

exploration of Dex effects. In particular, an experiment evaluating Dex concentration would provide valuable information. Since a comparison of the goat results to those in previous studies with human MPCs indicated that the response to Dex could be species-specific, it would be interesting to conduct experiments comparing chondrogenic differentiation of goat and human MPC donors. Additionally, while experiments in this thesis focused on differentiating MPC cultures, future work could focus on the long-term development of tissue-engineered implants using MPCs. The culture media currently used to support differentiation may not be ideal for extended *in vitro* growth of the MPC cultures. Identifying growth factors other than TGF-β1 or determining when serum could be included in the culture media are possible suggestions for enhancing the culture media. Use of bioreactors with MPC cultures will be another important consideration for long-term *in vitro* tissue development.

For the evaluation of mechanical loading influences on MPC chondrogenic activity, the effects of hydrogel and chondrogenic supplementation are issues which need further clarification. Conducting experiments with MPCs cultured in hydrogels containing cell-adhesion sequences would enable assessment of loading effects in an environment more representative of the *in vivo* situation. Modulating the TGF-β1 dose used to culture the MPCs might enable a more sensitive assessment of the relative influences of chemical and mechanical signals on the chondrogenic activity of differentiating cultures. As the loading frequency was shown to be important for changes in MPC biosynthesis in response to loading, evaluating loading influences at lower frequencies should be investigated. Additionally, the biochemical analyses utilized in the experiments on chondrogenic activity provided a lumped population response. Having an assay to assess

the response of individual cells would enable a more thorough evaluation of loading responses. One possible approach for future studies would be to evaluate isolated MPCs after loading with fluorescence activated cell sorting in combination with antibodies for cartilage matrix markers, with cell isolation achieved by dissolution of the MPC/alginate gels with calcium chelators.

In evaluating the mechanoresponsiveness of the MPCs as related to biosynthesis, timing effects associated with progression of chondrogenic differentiation are one area for additional exploration with the current culture system. It would be interesting to evaluate loading influences at culture times beyond day 7, in part to determine whether loading at higher frequencies is capable of stimulating biosynthesis in the MPCs cultures similar to the response observed in the BAC cultures at day 7. To clarify factors contributing to the response of the day 7 MPC cultures, an experiment could be conducted in which standard day 7 cultures are compared to modified day 7 cultures lacking matrix accumulation. To modify the day 7 cultures, MPCs could be isolated from alginate suspension, subjected to a mild collagenase digestion, and then reintroduced into alginate suspension. The influence of the hydrogel composition on the response to loading is another issue for further investigation. Comparing the response of the early MPC cultures in hydrogels with and without cell adhesion sequences would be useful.

In summary, this thesis work has provided an assessment of dynamic mechanical loading influences on MPC cultures undergoing chondrogenic differentiation. The results of this work provide an experimental foundation for studying oscillatory compressive mechanical loading effects on MPC/hydrogel cultures and have identified important experimental variables for further consideration. The information gained

through *in vitro* studies of differentiating MPC cultures will be important for increasing basic understanding of MPCs and may also prove valuable in guiding the future development of cartilage repair approaches.

APPENDIX A

GROWTH FACTOR EFFECTS ON CHONDROGENIC DIFFERENTIATION OF CANINE MESENCHYMAL PROGENITOR CELLS

While canine MPCs had been demonstrated to undergo *in vivo* osteochondrogenesis and *in vitro* osteogenesis [109], no reports had described *in vitro* chondrogenesis in these cells. This work characterized the ability of canine MPCs to undergo *in vitro* chondrogenic differentiation in response to growth factor supplementation during cell expansion and subsequent 3-D culture. FGF-2 was identified as a potent monolayer expansion factor influencing 3-D chondrogenic differentiation.

Methods

Materials

Calf thymus DNA was from Invitrogen Life Technologies, Chicago, IL. Hoechst 33258 dye, safranin-O, and fast green FCF were from Sigma-Aldrich, St. Louis, MO. Hyaluronidase was from Calbiochem, EMD Biosciences, San Diego, CA. For all other items not listed previously, the materials list in the Chapter 3 methods section details the vendors for these items and will not be repeated here in the interest of brevity.

Canine MPC Culture

Iliac crest bone marrow aspirates were obtained from six canine donors, processed by Percoll separation, and plated for adherent primary culture in collaboration with Osiris Therapeutics, Inc. [109]. Following primary culture, the cells were expanded for one (P1) or two (P2) consecutive passages (8000cells/cm^2) in low glucose DMEM containing 10% FBS and 1% antibiotic/antimycotic. In selected experiments, 1ng/ml FGF-2 was

included in the expansion media. A pellet culture system was utilized to promote chondrogenic differentiation of canine MPCs, based upon its previous use in differentiation studies with human and rabbit MPCs [11, 12, 153]. To initiate pellet cultures for 3D differentiation experiments, passaged MPCs were suspended in the appropriate chondrogenic media, $0.25x10^6$cells (0.5 ml cells at $0.5x10^6$cells/ml) were transferred to a 15 ml polypropylene conical tube, and the tubes wers centrifuged for 5 minutes at ~500-600g to yield a cell layer on the tube base. Within ~24 hours of culture, the cell layer detached from the tube base and contracted into a more rounded "pellet" shape. The base chondrogenic media was high glucose DMEM containing 1% ITS$^+$ premix, 1% antibiotic-antimycotic, 0.1mM non-essential amino acids, 40μg/ml L-proline, and 50μg/ml ascorbic acid-2-phosphate. Chondrogenic supplements evaluated were 100nM Dex and 10ng/ml TGF-β1. All cultures received fresh media changes every 2 days.

Experimental Design

The initial study to validate chondrogenic differentiation in canine MPC donors was divided into two separate experiments with three donors evaluated in each experiment. Both passage 1 and passage 2 cells were cultured as pellets. Pellets were cultured to 14 days in media supplemented with Dex alone or in combination with TGF-β1. Given the noted passage-dependency of chondrogenic differentiation for the positive donors identified in the first study, a second study was conducted to evaluate FGF-2 effects on MPC passaging and the subsequent chondrogenic differentiation potential. The second study consisted of one experiment with three donors evaluated. During monolayer expansion, MPCs were cultured in standard media or in media supplemented with FGF-2

for two passages. Both passage 1 and passage 2 cells were cultured as pellets. Pellets were cultured to day 14 in media supplemented with Dex alone or in combination with TGF-β1. At the designated harvest time, cultures were taken for biochemical analyses (n=3 per condition per donor) or for histological processing (n=2-3 per condition per donor) for each of the canine MPC studies.

Biochemical Analyses

All samples were rinsed in PBS, weighed, dried in a SpeedVac, and reweighed. Pellets were suspended in 100mM ammonium acetate pH 7.0 containing proteinase K (12.5μg/pellet) and incubated overnight at 60ºC. The digestion solution was analyzed for DNA and sulfated glycosaminglycan (sGAG) content. DNA content was determined using Hoechst 33258 dye and quantified against a calf thymus DNA standard [217]. 200μl dye **(**0.5μg/ml dye in 10mM Tris-HCl, 1mM Na_2EDTA, 100mM NaCl pH 7.4 buffer**)** was added to 10 μl sample in a 96 well plate. The mixture was incubated for ~15 minutes and then measured on a Perkin-Elmer HTS 7000plus plate reader (365nm excitation and 465nm emission). sGAG content was determined using DMMB dye and quantified against a chondroitin-4-sulfate standard from bovine cartilage [218]. Sample dilutions were prepared as necessary in the appropriate base digest buffer. 200μl dye (~16mg/ml dye in 41mM NaCl, 40mM glycine pH 3.0) was added to 20μl sample dilution in a 96 well plate and then measured immediately at 525nm on a Bio-Tek PowerWave X340 plate reader.

Histological Analyses

Intact pellets were rinsed in PBS, transferred to 10% neutral-buffered formalin for ~48 hours, rinsed in PBS, and then stored in 70% alcohol. Samples were subsequently

embedded in paraffin and sectioned at five microns for analysis. Histological analyses were performed using a standard Hematoxylin & Eosin stain and a safranin-O stain, which binds to sGAGs (see Appendix B for safranin-O protocol). An immunohistochemical assay was performed (see Appendix B for protocol) with a monoclonal antibody provided by the Developmental Studies Hybridoma Bank for Type II collagen (IgG II-II6B3).

Statistical Analysis

Data were analyzed using ANOVA (significance at $p<0.05$).

Results and Discussion

Initial canine donor validation experiments were conducted in high-density pellet culture. Six donors were evaluated at two expansion levels (passage one and passage two). For cultures supplemented with Dex and TGF-β1, three of six MPC donors (donors A02, WN8, and WN9) underwent chondrogenic differentiation in P1 cultures, while only donor WN9 underwent differentiation in P2 cultures (Figure 24). In contrast, supplementation with Dex alone did not support differentiation in any donor at either passage level. Chondrogenic differentiation was evidenced by elevated per cell sGAG accumulation, by positive safranin-O staining, and by positive type II collagen expression (Figure 25). A portion of Dex only cultures, as well as some Dex and TGF-β1 cultures which were lacking safranin-O staining, exhibited a faint positive staining for Type II collagen (not shown).

To further evaluate passaging effects on chondrogenic differentiation, monolayer expansion of MPCs in the presence of FGF-2 followed by pellet culture with Dex and TGF-β1 supplementation was evaluated in three donors. It was found that FGF-2

significantly enhanced differentiation in both P1 and P2 cultures supplemented with Dex and TGF-β1. Comparing cultures expanded with FGF-2 to control cultures, sGAG/DNA was significantly higher ($p<0.005$) and safranin-O positive matrix was more extensive for all groups expanded with FGF-2 except donor WN8 at passage 1 (Figure 26). In fact, passaging with FGF-2 promoted differentiation in donor A01, a donor which had failed previously to undergo differentiation at any passage. Pellets containing media supplemented with Dex only were also cultured and no chondrogenic differentiation was observed for either of the expansion conditions (data not shown).

This work identified FGF-2 as a potent monolayer expansion factor influencing subsequent 3-D chondrogenic differentiation. Canine MPC expansion with FGF-2 enhanced chondrogenic differentiation and matrix accumulation, similar to FGF-2 influences on human and rabbit MPCs [123, 219]. The extension of chondrogenic capacity to later passages noted in canine MPCs is also consistent with previous data for rabbit MPCs [123]. The interaction between FGF-2 and passaging indicates that more cells are able to undergo chondrogenic differentiation with exposure to FGF-2 than in control media. FGF-2 may modulate the MPC population as a whole or may selectively influence a subset of the proliferating population. FGF-2 could promote dividing MPCs to maintain an undifferentiated state to a greater extent than in control cultures. Alternatively, a subset of cells that are undifferentiated in the initial population could undergo enhanced proliferation in response to FGF-2, thereby increasing their progeny relative to the population as a whole.

Canine MPC chondrogenic differentiation was found to be both donor- and passage-dependent. The donor variability observed in this study could reflect underlying

differences in the cell population present in different donors, but could also indicate variability introduced by the MPC harvesting technique. Previous work with canine MPCs observed passage dependency during *in vitro* osteogenesis, and this loss of *in vitro* differentiation with passaging appears to be accelerated in canine MPC populations compared to other species [12, 109]. The demonstration of notable donor variability in the canine MPCs led to the conclusion that these cells were not an appropriate choice for the thesis work. However, the canine MPC work identified FGF-2 as a beneficial monolayer expansion factor influencing 3-D chondrogenic differentiation, and FGF-2 was included as a monolayer expansion supplement in the goat MPC cultures.

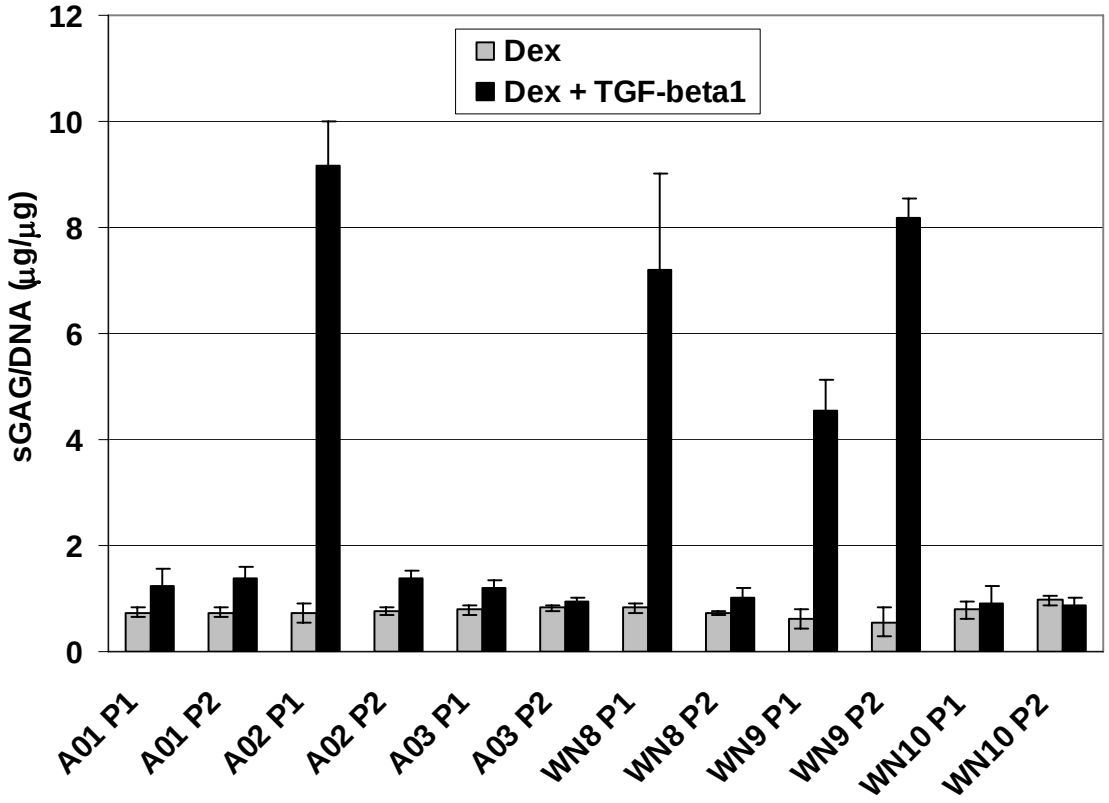


Figure 24: Canine MPC Donor Comparison of Day 14 sGAG/DNA Content. Pellets cultures were evaluated for 6 donors (i.e. donor A01) at passage 1 (P1) and passage 2 (P2). Pellets were grown in media supplemented with Dex only or with both Dex and TGF-β1. Bars represent mean ± standard deviation (n=3pellets/donor; 1 experiment).

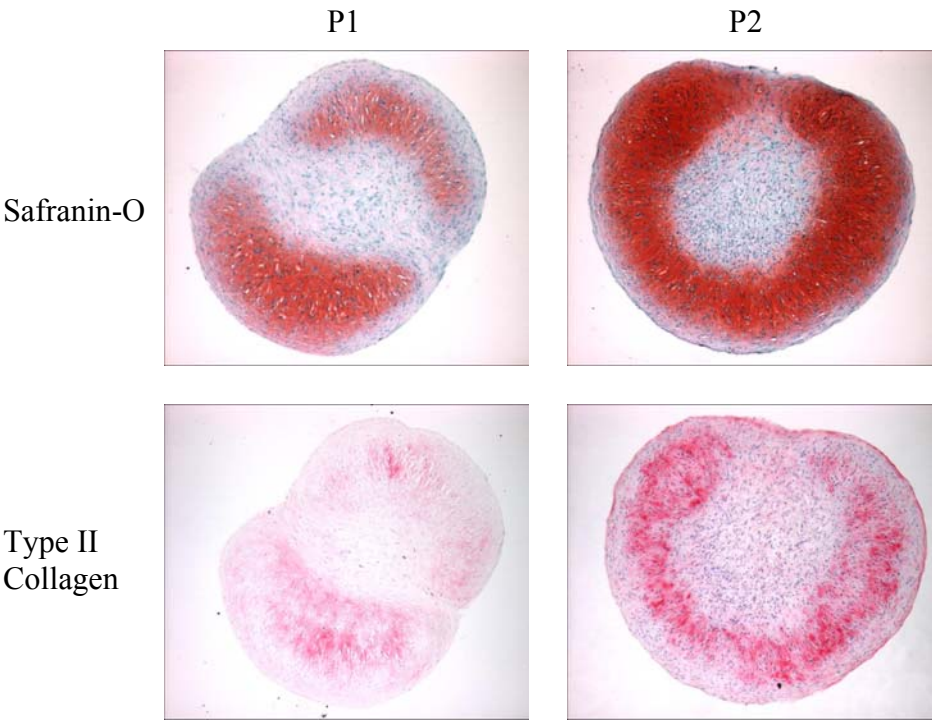


Figure 25: Histological Analyses of Day 14 Canine MPC Pellets. Cultures were supplemented with Dex & TGF-β1. Safranin-O staining (10X, red coloration indicates presence of sGAGs) is shown on top and Type II collagen immunostaining (10X, red coloration indicates presence of antigen) is shown on bottom. Images are shown for donor WN9.

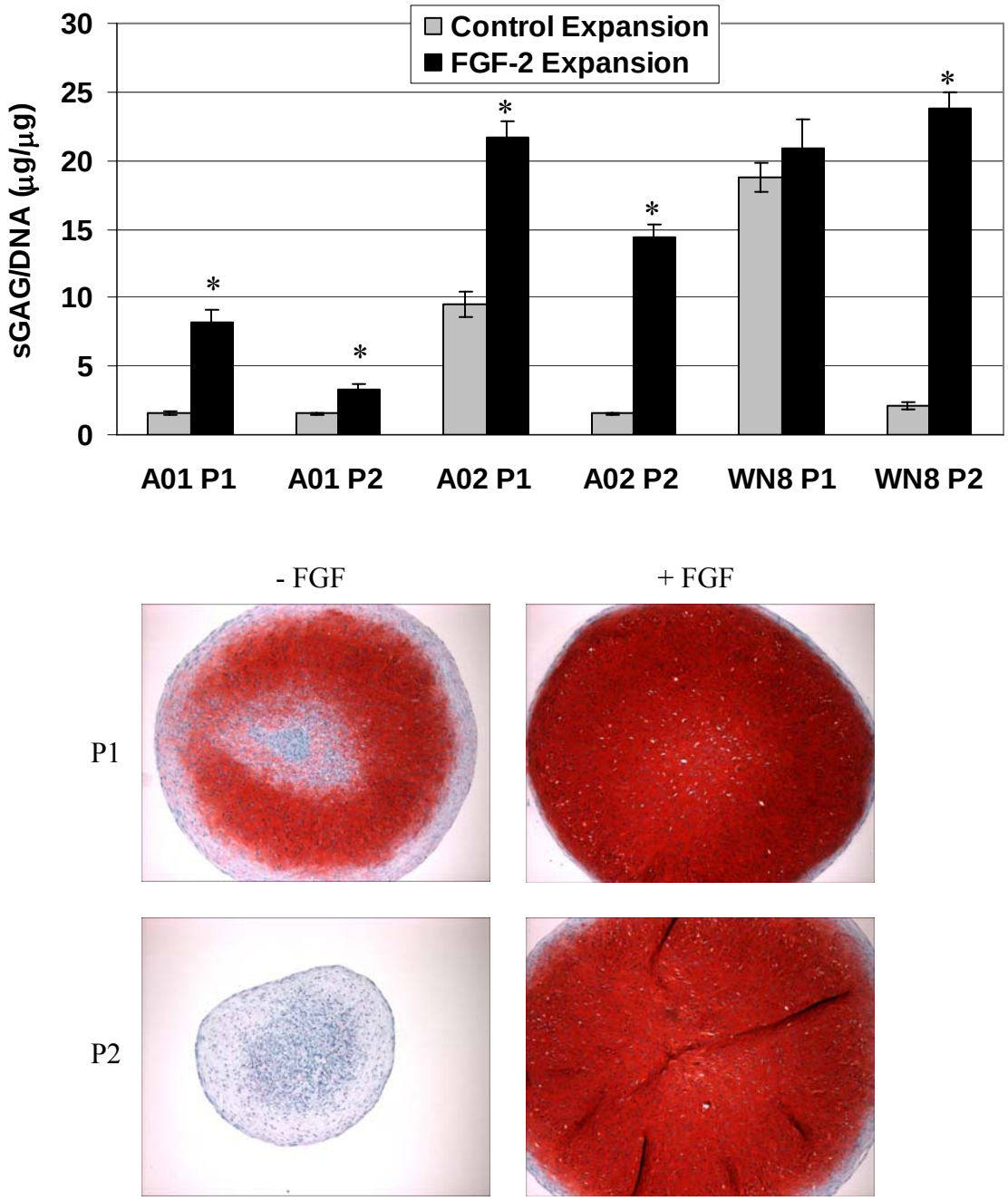


Figure 26: Chondrogenic Differentiation in Response to FGF-2 Supplementation during Monolayer Expansion. All pellet cultures received Dex and TGF-β1. Top: Comparison of day 14 sGAG/DNA content. Bars represent mean ± standard deviation (n=3pellets/donor). * denotes significantly different versus control expansion for the designated donor/passage. Bottom: Safranin-O staining (10X, red coloration indicates presence of sGAGs) of P1 and P2 day 14 cultures for donor A02.

APPENDIX B

IMMUNOHISTOCHEMICAL AND HISTOLOGICAL PROTOCOLS

I. Immunohistochemistry for Type II Collagen Staining

1. For paraffin sections (≤ 5micron thickness), follow standard deparaffinization procedure to water.
2. Rinse 5 minutes in 1X PBS.
3. Apply pepsin solution to tissue sections and incubate 10 minutes, then repeat with fresh pepsin solution.
4. Rinse 5 minutes in 1X PBS and repeat rinse.
5. Cover tissue sections with 1% gelatin/PBS for 20 minutes.
6. Apply diluted (1:4 in 1%BSA/1X PBS) primary antibody (IgG II-II6B3, Developmental Studies Hybridoma Bank) to tissue sections and incubate 1 hour.
7. Tap off excess antibody, rinse 5 minutes in 1X PBS, and repeat rinse.
8. Apply diluted (1:400 in 1%BSA/1X PBS additionally containing 2% serum) secondary antibody (Anti-Mouse IgG, Vector Labs #BA-2001) to tissue sections and incubate 30 minutes.
9. Prepare ABC solution by adding 1 drop each of reagent A and B (Vector Labs #AK-5000) to 5ml 1XPBS and let sit 30 minutes.
10. Tap off excess antibody, rinse 5 minutes in 1X PBS, and repeat rinse.
11. Apply ABC solution to tissue sections and incubate 1 hour.
12. Rinse 5 minutes in 1X PBS and repeat rinse.
13. Rinse 5 minutes in 100mM Tris pH 8.2.
14. Immediately before using prepare vector red substrate in container wrapped in aluminum foil: To 5ml 100mM Tris pH 8.2 add 1 drop levamisole (Vector Labs #SP-5000) and 2 drops each reagents 1, 2, & 3 (Vector Labs #SK-5100) and mix by inversion.
15. Apply substrate to tissue sections and incubate up to 45 minutes in dark, checking periodically for color development.
16. Rinse 5 minutes in tap water and repeat rinse.
17. Counterstain slides with hematoxylin as per standard procedure.

II. Safranin-O/Fast Green/Weigert's Iron Hematoxylin Stain

1. For paraffin sections (≤ 5micron thickness), deparaffinize to water: Xylene for 3 minutes and repeat two times; 100% Alcohol for 2 minutes and repeat two times; 95% Alcohol for 2 minutes; water rinse
2. Deionized water for 1 minute
3. Weigert's Iron Hematoxylin Working Solution for 1-2 minutes (prepared by combining equal volumes of Part A and Part B solutions and then filtering using Whatman filter paper; Sigma #HT10-79)
4. Running tap water rinse for 2 minutes

5. One dip in 1% acid alcohol (1 volume 12N HCl in 99 volumes 70% Alcohol)
6. Running tap water rinse for 2 minutes
7. 0.02% Fast Green Solution for 1 minute (prepared by adding 0.02 grams Fast Green FCF powder, Sigma Catalog #F7258, to 100 mls deionized water)
8. 1% acetic acid for 3 seconds
9. 6% Safranin-O solution (Sigma #HT90-4-32) for 5 minutes
10. Dehydrate slides then coverslip: 95% Alcohol for 1 minute; 100% Alcohol for 10 seconds and repeat two times; Xylene for 10 seconds and repeat two times

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